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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

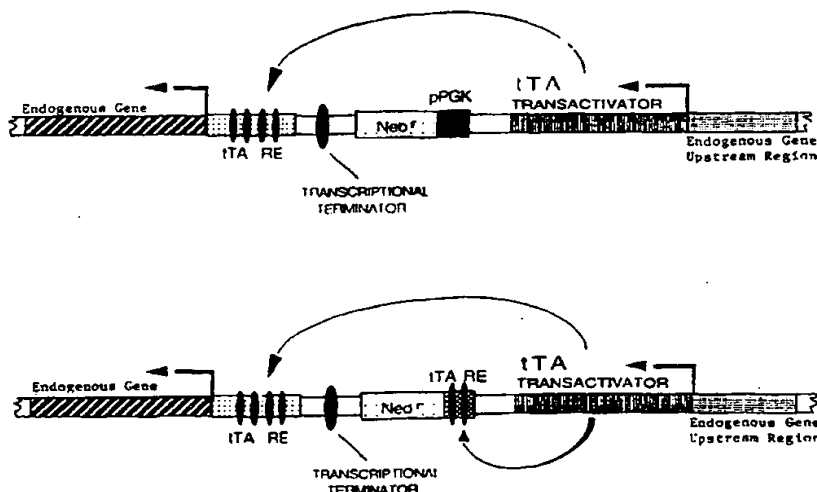
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(71) Applicant (for all designated States except US): BASF AK- TIENGESELLSCHAFT [DE/DE]; D-67056 Ludwigshafen (DE).			
(72) Inventors; and (75) Inventors/Applicants (for US only): BUJARD, Hermann [DE/DE]; Remler Strasse 9, D-69120 Heidelberg (DE). GOSSEN, Manfred [DE/DE]; Im Neuenheimer Feld 282, D-69120 Heidelberg (DE). SALFELD, Jochen, G. [DE/US]; 177 Old Westboro Road, North Grafton, MA 01536 (US). VOSS, Jeffrey, W. [US/US]; Apartment 10, 35 Georgetown Road, Framingham, MA 01701 (US).			

(54) Title: TIGHT CONTROL OF GENE EXPRESSION IN EUKARYOTIC CELLS BY TETRACYCLINE-RESPONSIVE PROMOTERS

## (57) Abstract

Transgenic animals carrying two transgenes, the first coding for a transactivator fusion protein comprising a tet repressor and a polypeptide which directly or indirectly activates in eucaryotic cells, and the second comprising a gene operably linked to a minimal promoter operably linked to at least one tet operator sequence, are disclosed. Isolated DNA molecules (e.g., targeting vectors) for integrating a polynucleotide sequence encoding a transactivator of the invention at a predetermined location within a second target DNA molecule by homologous recombination are also disclosed. Transgenic animals having the DNA molecules of the invention integrated at a predetermined location in a chromosome by homologous recombination are also encompassed by the invention. Methods to regulate the expression of a tet operator linked gene of interest by administering tetracycline or a tetracycline analogue to an animal of the invention are also disclosed. The regulatory system of the invention allows for conditional inactivation or modulation of expression of a gene of interest in a host cell or animal.

## Conditional Knock-Out Strategy 3



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**Tight Control of Gene Expression in Eucaryotic Cells  
by Tetracycline-Responsive Promoters**

5

**Background of the Invention**

The study of gene function in complex genetic environments such as eucaryotic cells would greatly profit from systems that would allow stringent control of the expression of individual genes. Ideally, such systems would not only mediate an "on/off" status for gene  
10 expression but would also permit limited expression at a defined level.

Attempts to control gene activity by various inducible eucaryotic promoters responsive to, for example, heavy metal ions (Mayo et al., Cell 29:99-108 (1982); Brinster et al., Nature (London) 296:39-42 (1982); Searle et al., Nouer, L., CRC Boca Raton, FL (1991), pp. 167-220), or hormones (Lee et al., Nature (London) 294:228-232 (1981); Hynes et al.,  
15 Proc. Natl. Acad. Sci. USA 78:2038-2042 (1981); Klock et al., Nature (London) 329:734-736 (1987); Israel & Kaufman, Nucleic Acids Res. 17:2589-2604 (1989)) have generally suffered from leakiness of the inactive state (e.g., the metallothionein promoter (Mayo et al., Cell 29:99-108 (1982)) or from pleiotropic effects caused by the inducing principles themselves, such as elevated temperature or glucocorticoid hormone action (Lee et al., Proc. Natl. Acad.  
20 Sci, USA 85:1204-1208 (1988)).

In search of regulatory systems that do not rely on endogenous control elements, several groups have demonstrated that the lac repressor/operator inducer system of Escherichia coli functions in eucaryotic cells. Three approaches have been described: (i) prevention of transcription initiation by properly placed lac operators at promoter sites (Hu &  
25 Davidson, Cell 48:555-566 (1987); Brown et al., Cell 49:603-612 (1987); Figge et al., Cell 52:713-722 (1988); Fuerst et al., Proc. Natl. Acad. Sci. USA 86:2549-2553 (1989); Deutschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5405 (1989)), (ii) blockage of transcribing RNA polymerase II during elongation by a lac repressor/operator complex (lac R/O; Deutschle et al., Science 248:480-483 (1990)), and (iii) activation of a promoter responsive to a fusion  
30 between lacR and the activating domain of virion protein 16 (VP16) of herpes simplex virus (HSV) (Labow et al., Mol. Cell. Biol. 10:3343-3356 (1990); Baim et al., Proc. Natl. Acad. Sci. USA 88:5072-5076 (1991)).

At present, however, the utility of the lacR/O-based systems in eucaryotic cells is limited since the inducer isopropyl.β-D-thiogalactopyranoside (IPTG), despite its rapid  
35 uptake and intracellular stability (Wyborski & Short, NucleicAcids Res. 19:4647-4653), acts rather slowly and inefficiently, resulting in only moderate induction. Nevertheless, an interesting conditional mutant of a lacR-VP16 fusion has been described (Baim et al., Proc. Natl. Acad. Sci. USA 88:5072-5076 (1991)). It activates a minimal promoter ~ 1000-fold at elevated temperatures in the presence of IPTG. The temperature dependence and the inherent

IPTG-related problems, however, may also limit this approach.

### Summary of the Invention

This invention features a system for regulating expression of eucaryotic genes using components of the Tet repressor/operator/ inducer system of prokaryotes. In the system of the invention, transcription of a nucleotide sequence operably linked to at least one *tet* operator sequence is stimulated by a tetracycline (Tc)-controllable transcriptional activator fusion protein (referred to herein as tTA). The tTA is comprised of two polypeptides. The first polypeptide is a Tet repressor (TetR; e.g., a Tn10-derived TetR), which binds to *tet* operator sequences in the absence but not the presence of Tc. The second polypeptide directly or indirectly activates transcription in eucaryotic cells. For example, the second polypeptide can be a transcriptional activation domain from herpes simplex virus virion protein 16 or another transcriptional activating domain, e.g. acidic, proline-rich, serine/threonine-rich, glutamine-rich. Alternatively, the second polypeptide can be a domain (e.g., a dimerization domain) which recruits a transcriptional activator (e.g., an endogenous transcriptional activator) to interact with the tTA fusion protein by a protein-protein interaction (e.g., a non-covalent interaction). In the absence of Tc or a Tc analogue, transcription of a gene operably linked to a tTA-responsive promoter (typically comprising at least one *tet* operator sequence and a minimal promoter) is stimulated by a tTA of the invention, whereas in the presence of Tc or a Tc analogue, transcription of the gene linked to the tTA-responsive promoter is not stimulated by the tTA.

As described herein, this system functions effectively in transgenic animals. Accordingly, the invention provides a tetracycline-controllable regulatory system for modulating gene expression in transgenic animals. Additionally, the invention provides targeting vectors for homologous recombination that enable the components of the regulatory system to be integrated at a predetermined location in the genome of a host cell or animal. This embodiment of the invention is able to solve a longstanding problem in the field generally described as gene targeting or gene knock out. Constitutive disruption of certain genes has been found to produce lethal mutations resulting in death of homozygous embryos, e.g., as described for the knock out of the RB gene (Jacks, T. et al. (1992) Nature 359:295-300). This problem precludes the development of "knock out" animals for many genes of interest. The regulatory system of the invention can be applied to overcome this problem. DNA encoding a tTA of the invention can be integrated within a gene of interest such that expression of the tTA is controlled by the endogenous regulatory elements of the gene of interest (e.g., the tTA is expressed spatially and temporally in a manner similar to the gene of interest). The gene of interest is then operably linked to at least one *tet* operator sequence (either at its endogenous site by homologous recombination or a second copy of the gene of interest, linked to *tet* operator(s), can be integrated at another site). Expression of the *tet*-operator linked gene is thus placed under the control of the tTA, whose pattern of

expression mimics that of the gene of interest. In the absence of Tc, expression of the tet operator-linked gene of interest is stimulated by the tTA and the animal develops like a nonmutated wildtype animal. Then, at a particular stage of development, expression of the gene of interest can be switched off by raising the level of Tc (or a Tc analogue) in the circulation and the tissues of the animal by feeding or injecting Tc (or a Tc analogue) to the animal, thereby inhibiting the activity of the tTA and transcription of the gene of interest. This method is generally referred to herein as a "conditional knockout".

Accordingly, one aspect of the invention relates to targeting vectors for homologous recombination. In one embodiment, the invention provides an isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) at a predetermined location in a second target DNA molecule. In this DNA molecule, a polynucleotide sequence encoding a tTA is flanked at 5' and 3' ends by additional polynucleotide sequences of sufficient length for homologous recombination between the DNA molecule and the second target DNA molecule at a predetermined location. Typically, the target DNA molecule into which the tTA-coding sequences are integrated is a gene of interest, or regulatory region thereof, in a eucaryotic chromosome in a host cell. For example, tTA-coding sequences can be inserted into a gene within a yeast, fungal, insect or mammalian cell. Additionally, tTA-coding sequences can be inserted into a viral gene present within a host cell, e.g. a baculovirus gene present in insect host cell. In a preferred embodiment, integration of the tTA-encoding sequences into a predetermined location in a gene of interest (by homologous recombination) places the tTA-coding sequences under the control of regulatory elements of the gene of interest (e.g., 5' flanking regulatory elements), such that the tTA is expressed in a spatial and temporal manner similar to the gene of interest.

In another embodiment of the targeting vector for homologous recombination, the isolated DNA molecule permits integration of a polynucleotide sequence encoding both a tTA and a tTA-responsive promoter within a predetermined gene of interest in a second target DNA molecule (a "single hit vector", schematically illustrated in Figure 13A-B). This molecule includes: 1) a first polynucleotide sequence comprising a 5' flanking regulatory region of the gene of interest, operably linked to 2) a second polynucleotide sequence encoding a tTA; and 3) a third polynucleotide sequence comprising a tTA-responsive promoter, operably linked to: 4) a fourth polynucleotide sequence comprising at least a portion of a coding region of the gene of interest. The first and fourth polynucleotide sequences are of sufficient length for homologous recombination between the DNA molecule and the gene of interest such that expression of the tTA is controlled by 5' regulatory elements of the gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter (i.e., upon binding of the tTA to the tTA-responsive promoter, expression of the gene of interest is stimulated). This targeting vector can also include a polynucleotide sequence encoding a selectable marker operably linked to a regulatory sequence. Typically, the selectable marker expression unit is located between the tTA-

encoding sequence (i.e., the second polynucleotide sequence described above) and the tTA-responsive promoter (i.e., the third polynucleotide sequence described above). Additionally or alternatively, this targeting vector can also include a sequence, typically located upstream (i.e., 5') of the tTA-responsive promoter (e.g., between the selectable marker expression unit and the tTA responsive promoter) which terminates transcription or otherwise insulated downstream elements from the effects of upstream regulatory elements. The tTA-responsive promoter typically includes a minimal promoter operably linked to at least one tet operator sequence. The minimal promoter is derived, for example, from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.

Another aspect of the invention relates to eucaryotic host cells containing a DNA molecule encoding a tTA integrated at a predetermined location in a second target DNA molecule (e.g., a gene of interest) in the host cell. Such a eucaryotic host cell can be created by introducing a targeting vector of the invention into a population of cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the second target DNA molecule and selecting a cell in which the DNA encoding the tTA has integrated at a predetermined location within the second target DNA molecule. The host cell can be a mammalian cell (e.g., a human cell). Alternatively, the host cell can be a yeast, fungal or insect cell (e.g., the tTA-encoding DNA can be integrated into a baculovirus gene within an insect cell). A preferred host cell type for homologous recombination is an embryonic stem cell, which can then be used to create a non-human animal carrying tTA-coding sequences integrated at a predetermined location in a chromosome of the animal. A host cell can further contain a gene of interest operably linked to a tTA-responsive transcriptional promoter. The gene of interest operably linked to the tTA-responsive promoter can be integrated into DNA of the host cell either randomly (e.g., by introduction of an exogenous gene) or at a predetermined location (e.g., by targeting an endogenous gene for homologous recombination). The gene linked to the tTA-responsive promoter can be introduced into the host cell independently from the DNA encoding the tTA, or alternatively, a "single hit" targeting vector of the invention can be used to integrate both tTA-coding sequences and a tTA-responsive promoter into a predetermined location in DNA of the host cell. Expression of a gene of interest operably linked to a tTA-responsive promoter in a host cell of the invention can be inhibited by contacting the cell with tetracycline or a tetracycline analogue.

Another aspect of the invention relates to non-human transgenic animals having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) of the invention or having a transgene encoding a gene of interest operably linked to a tTA-responsive promoter. Double transgenic animals having both transgenes (i.e., a tTA-coding transgene and a gene of interest linked to a tTA-responsive promoter) are also encompassed by the invention. In one embodiment, the transgenic animal is a mouse. In other embodiments, the transgenic animal is a cow, a goat, a sheep and a pig. Transgenic animals of the invention can be made, for example, by introducing a DNA



molecule encoding the tTA or the gene of interest operably linked to a tTA responsive promoter into a fertilized oocyte, implanting the fertilized oocyte in a pseudopregnant foster mother, and allowing the fertilized oocyte to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal. Double transgenic animals can be  
5 created by appropriate mating of single transgenic animals. Expression of a gene of interest operably linked to a tTA responsive promoter in a double transgenic animal of the invention can be inhibited by administering tetracycline or a tetracycline analogue to the animal.

Another aspect of the invention relates to non-human transgenic animals having a transgene encoding a tTA of the invention, wherein the transgene is integrated by  
10 homologous recombination at a predetermined location within a chromosome within cells of the animal (also referred to herein as a homologous recombinant animal). The homologous recombinant animal can also have a second transgene encoding a gene of interest operably linked to a tTA-responsive promoter. The second transgene can be introduced randomly or, alternatively, at a predetermined location within a chromosome (e.g., by homologous  
15 recombination. For example, a single hit vector of the invention can be used to create a homologous recombinant animal in which expression of the tTA is controlled by 5' regulatory elements of a gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter (such that in the absence of Tc, expression of the gene is stimulated by tTA binding to the tTA responsive promoter).

20 A non-human transgenic animal of the invention having tTA-coding sequences integrated at a predetermined location within chromosomal DNA of cells of the animal can be created by introducing a targeting vector of the invention into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and chromosomal DNA within the cell, selecting an embryonic stem cell in which  
25 DNA encoding the tTA has integrated at a predetermined location within the chromosomal DNA of the cell, implanting the embryonic stem cell into a blastocyst, implanting the blastocyst into a pseudopregnant foster mother and allowing the blastocyst to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.

Another aspect of the invention relates to a process for producing and isolating a gene  
30 product (e.g., protein) encoded by a gene of interest operably linked to a tTA-responsive transcriptional promoter in a host cell of the invention carrying tTA-coding sequences. In the process, a host cell is first grown in a culture medium in the presence of tetracycline or a tetracycline analogue (under these conditions, expression of the gene of interest is not stimulated). Next, the concentration of tetracycline or the tetracycline analogue in the culture  
35 medium is reduced to stimulate transcription of the gene of interest. The cells are further cultured until a desired amount of the gene product encoded by the gene of interest is produced by the cells. Finally, the gene product is isolated from harvested cells or from the culture medium. Preferred cells for use in the process include yeast or fungal cells.

Kits containing the components of the regulatory system of the invention described

herein are also within the scope of the invention.

Various additional features, components and aspects of this invention are described in further detail below.

## 5 **Brief Description of the Figures**

**Fig. 1 (panels a and b).** Schematic representation of tetR-VP16 fusion proteins (tTAs), encoded by plasmids pUHD15-1 and pUHD151-1, and a tTA-dependent transcription unit, encoded by plasmid pUHC13-3.

**Panel a:** Diagrammatic representation of two tTA proteins. In both fusion proteins, 10 tTA and tTAS, the original 207-amino-acid sequence of tetR is conserved. Two versions of VP 16 sequences encoding the activation domain were fused in frame to the 3' end of the tetR gene, resulting in tTA and tTAS. The bold letters indicate the original amino acids at the N terminal end, the junction, and the C-terminal end of the fusion proteins; the other letters designate amino acids introduced due to sequence constraints of the particular system. The 15 numbers delineate amino acid positions within tetR (Hillen and Wissman in Protein-Nucleic Acid Interaction, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Vol 10, pp. 143-162 (1989)) or VP16 (Treizenberg et al., Genes Dev. 2:718-729 (1988)), respectively.

**Panel b:** The tTA-dependent transcriptional unit consists of the simian virus 40 20 (SV40) poly(A) site (An), the luciferase gene (luc), the PhCMV\*-1 or PhCMV\*-2. The two promoters encompass the sequence between +75 and -53 of the PhCMV\*-2 with one base-pair exchange at -31, which creates a Stu I cleavage site. The Xho I site introduced at -53 by PCR was utilized to insert the heptamerized tetO sequence. This heptameric sequence is flanked at one side by an 18-nucleotide polylinker, which allows the insertion of the 25 operators in both orientations as Sal I/XhoI fragments. The position of the central G/C base pair of the promoter proximal operator to position +1 is -95 for PhCMV\*-1 (upper construct) and -76 for PhCMV\*-2 (lower construct). The plasmids that contain the four constructs are indicated on the far right.

**Fig. 2 (panels a and b).** Western blots showing the identification and characterization 30 of tTA produced in HeLa cells. HeLa cells grown to 40% confluency were transiently transfected with pUHD15-1 by the calcium phosphate method. Nuclear and cytoplasmic extracts were prepared after 36 hr.

**Panel a:** Western blot analysis of electrophoretically separated extracts (6% 35 acrylamide/0.1% SDS gels) with tetR-specific antibodies reveals a protein of about 37 kDa (tTA) in cytoplasmic (C) and nuclear (N) extracts in pUHD15-1 transfected cells (+) that is not present in mock-transfected cells (-).

**Panel b:** Mobility change of tetO DNA by tTA binding from HeLa cell nuclear extracts. Radioactively labeled tetO DNA was mixed with extracts from mock-transfected (lanes 2 and 3) and pUHD15-1-transfected (lanes 4 and 5) HeLa cells in the absence (lanes 2

and 4) and presence (lanes 3 and 5) of 1  $\mu$ g of tetracycline per ml (added 2 min prior to the addition of the operator). Lane 1 contains labeled operator DNA only.

Fig. 3 (panels a and b). Graphs showing the dependence of tTA function on tetracycline.

5 Panel a: Dependence of luciferase (luc.) activity on tetracycline concentration. HeLa cell clones X1 (dashed line) and T12 previously grown in tetracycline-free medium were seeded with a density of 5000 cells per 35mm dish and incubated at the tetracycline concentrations indicated. After reaching ~ 90% confluency, cells were harvested and assayed for luciferase activity. Data given are the means  $\pm$  SD of three independent experiments.

10 Panel b: Kinetics of tetracycline action. X1 cells were grown in 100mm dishes to ~ 80% confluency in the absence or presence (0.1  $\mu$ g/ml) of tetracycline. At time 0, cells were washed with phosphate-buffered saline and split into smaller culture dishes (1/20th of the initial cultures per 35mm dish). Half of the cultures remained in tetracycline-free medium (■) and the other half were incubated in the presence of tetracycline (1  $\mu$ g/ml; □). The X1  
15 culture grown in tetracycline-containing medium was split in the same manner; one half was further incubated in the presence of tetracycline (●), whereas the other half was transferred to tetracycline-free medium (○). At the times indicated, aliquots were harvested and examined for luciferase activity. The slight increase in luciferase activity monitored at 4 hr in the culture containing tetracycline (●) is reproducible and reflects luciferase induction during the  
20 washing step.

Fig. 4. [SEQ ID NO: 1] The polynucleotide sequence coding for tTA transactivator.

Fig. 5. [SEQ ID NO: 3] The polynucleotide sequence coding for tTA<sub>S</sub> transactivator.

Fig. 6. [SEQ ID NO: 5] The polynucleotide sequence of P<sub>hCMV</sub>\*-1. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the hCMV minimal promoter, of which position -53, the TATA box and position +75 (relative to the transcription  
25 start site) are underlined.

Fig. 7. [SEQ ID NO: 6] The polynucleotide sequence of P<sub>hCMV</sub>\*-2. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the hCMV minimal promoter, of which position -53, the TATA box and position +75 (relative to the transcription  
30 start site) are underlined .

Fig. 8. [SEQ ID NO: 7] The polynucleotide sequence of P<sub>Tk</sub>\*-1. The nucleotide sequence shown encompasses the tet operator sequences (*italics*) and the HSV-Tk minimal promoter, of which position -81, the TATA box and position +7 (relative to the transcription start site) are underlined .

35 Fig. 9A-9C. [SEQ ID NO: 8] The polynucleotide sequence of the cDNA coding for the rabbit progesterone receptor under control of P<sub>hCMV</sub>\*-1

Fig. 10A-B. [SEQ ID NO: 9] The polynucleotide sequence of the cDNA coding for the rabbit progesterone receptor under control of P<sub>hCMV</sub>\*-1

Fig. 11. A schematic representation of Conditional Knock Out Strategy 1 in which

"E.G. 5' " represents flanking nucleotide sequence from 5' of the coding sequence for an Endogenous Gene; "E.G. 3' " represents flanking nucleotide sequence from 3' of the coding sequence for an Endogenous Gene; and "tTARE" represents a tTA responsive element inserted just upstream of a copy of the endogenous gene of interest. (In other embodiments the gene linked to the tTA is a heterologous gene.)

Fig. 12. A schematic representation of Conditional Knock Out Strategy 2 in which "tTARE" is a tTA responsive promoter element; "E.G." is an endogenous gene; "E.G. 5' " represents flanking nucleotide sequence from 5' of the coding sequence for an Endogenous Gene; "E.G. 3' " represents flanking nucleotide sequence from 3' of the coding sequence for an Endogenous Gene; and "TK" is a thymidine kinase gene.

Fig. 13.A-B A schematic representation of Conditional Knock Out Strategy 3 depicting vector designs in which abbreviations are as defined above, Neo<sup>r</sup> is a neomycin resistance gene and pPGK is phosphoglycerate kinase sequence.

Fig. 14. A graphic representation of the doxycycline dependent luciferase activity in double transgenic mice carrying P<sub>h</sub>CMV-tTA and P<sub>h</sub>CMV\*-1-luc transgenes. Light bars show tTA-activated luciferase levels in different tissues from 2 individual mice. Dark bars show luciferase levels in different tissues from 2 individual mice that received doxycycline in the drinking water (200 mg/ml, 5 % sucrose) for 7 days. Spotted bars (controls) represent the average luciferase background activity from 5 individuals from line L7, carrying only the P<sub>h</sub>CMV\*-1-luc transgene.

## **Detailed Description of the Invention**

### **Definitions**

The description that follows makes use of a number of terms used in recombinant DNA technology. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Cloning Vector.** A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector.

**Expression Vector.** A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either

constitutive or inducible.

Eucaryotic Host Cell. According to the invention, a eucaryotic host cell may be any such cell which include, but are not limited to, yeast cells, plant cells, fungal cells, insect cells, e.g. Schneider and sF9 cells, mammalian cells, e.g. HeLa cells (human), NIH3T3  
5 (murine), RK13 (rabbit) cells, embryonic stem cell lines, e.g. D3 and J1, and cell types such as hematopoietic stem cells, myoblasts, hepatocytes, lymphocytes, airway epithelium and skin epithelium.

Recombinant Eucaryotic Host. According to the invention, a recombinant eucaryotic host may be any eucaryotic cell which contains the polynucleotide molecules of the present  
10 invention on an expression vector or cloning vector. This term is also meant to include those eucaryotic cells that have been genetically engineered to contain the desired polynucleotide molecules in the chromosome, genome or episome of that organism. Thus, the recombinant eucaryotic host cells are capable of stably or transiently expressing the proteins.

Recombinant vector. Any cloning vector or expression vector which contains the  
15 polynucleotide molecules of the invention.

Host. Any prokaryotic or eucaryotic cell that is the recipient of a replicable vector. A "host" as the term is used herein, also includes prokaryotic or eucaryotic cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, see Sambrook et al., Molecular  
20 Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

Promoter. A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription  
25 increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Minimal Promoter. A partial promoter sequence which defines the transcription start site but which by itself is not capable, if at all, of initiating transcription efficiently. The activity of such minimal promoters depend on the binding of activators such as a  
30 tetracycline-controlled transactivator to operably linked binding sites.

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein.

Structural Gene. A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Polynucleotide molecules. A polynucleotide molecule may be a  
35 polydeoxyribonucleic acid molecule (DNA) or a polyribonucleic acid molecule (RNA).

Complementary DNA (cDNA). A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Expression. "Expression" is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

5 Fragment. A "fragment" of a molecule is meant to refer to any polypeptide subset of that molecule.

Tet repressor. A "tet repressor" refers to a prokaryotic protein which binds to a tet operator sequence in the absence but not the presence of tetracycline. The term "tet repressor" is intended to include repressors of different class types, e.g., class A, B, C, D or E tet repressors.

10 Tetracycline Analogue. A "tetracycline analogue" is any one of a number of compounds that are closely related to tetracycline (Tc) and which bind to the tet repressor with a  $K_a$  of at least about  $10^6 \text{ M}^{-1}$ . Preferably, the tetracycline analogue binds with an affinity of about  $10^9 \text{ M}^{-1}$  or greater, e.g.  $10^9 \text{ M}^{-1}$ . Examples of such tetracycline analogues include, but are not limited to those disclosed by Hlavka and Boothe, "The Tetracyclines," in  
15 Handbook of Experimental Pharmacology 78, R.K. Blackwood et al. (eds.), SpringerVerlag, Berlin-New York, 1985; L.A. Mitscher "The Chemistry of the Tetracycline Antibiotics, Medicinal Research 9, Dekker, New York, 1978; Noyee Development Corporation, "Tetracycline Manufacturing Processes," Chemical Process Reviews, Park Ridge, N.J., 2 volumes, 1969; R.C. Evans, "The Technology of the Tetracyclines," Biochemical Reference  
20 Series 1, Quadrangle Press, New York, 1968; and H.F. Dowling, "Tetracycline," Antibiotics Monographs, no. 3, Medical Encyclopedia, New York, 1955; the contents of each of which are fully incorporated by reference herein. Examples of tetracycline analogues include anhydrotetracycline, doxycycline, chlorotetracycline, epioxytetracycline, and the like. Certain Tc analogues, such as anhydrotetracycline and epioxytetracycline, have reduced  
25 antibiotic activity compared to Tc.

Transgenic Animal. A transgenic animal is an animal having cells that contain a transgene, wherein the transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome  
30 of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. Non-human animals into which transgenes can be introduced by techniques known in the art include mice, goats, sheep, pigs, cows and other domestic farm animals.

A transgenic animal can be created, for example, by introducing a nucleic acid  
35 encoding a protein of interest (typically linked to appropriate regulatory elements, such as a constitutive or tissue-specific enhancer) into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. Methods for generating transgenic

animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan, B. et al., (1986) *A Laboratory Manual*, Cold Spring Harbor, New York, Cold Spring Harbor Laboratory. A transgenic founder animal can be used to breed additional animals carrying the transgene. A transgenic animal carrying one transgene can further be bred to another transgenic animal carrying a second transgenes to create a so-called "double transgenic" animal carrying two transgenes.

Homologous Recombinant Animal. The term "homologous recombinant animal" as used herein is intended to describe an animal containing a gene which has been modified by homologous recombination between the gene and a DNA molecule introduced into an embryonic cell of the animal, or ancestor thereof. Thus, a homologous recombinant animal is a type of transgenic animal in which the transgene is introduced into a predetermined chromosomal location in the genome of the animal by homologous recombination.

To create such a homologous recombinant animal, a vector is prepared which contains DNA of interest (e.g., encoding a tTA of the invention) flanked at its 5' and 3' ends by additional nucleic acid of a eucaryotic gene of interest at which homologous recombination is to occur. The flanking nucleic acid is of sufficient length for successful homologous recombination with the eucaryotic gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harbouring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by so-called "germline transmission". Animals carrying the recombined gene can be bred to homozygosity and/or bred with other animals carrying other transgenes.

Recombinant expression of proteins is commonly done using constitutive promoters like human CMV (Boshart, M. et al. 1985, *Cell* Vol. 41, 521-530) or the adenovirus major late promoter or SV40 early promoter as described below (see also, e.g., Kaufman, R.J. 1990 *Meth Enzymol.* Vol. 185: 537-566 and Benoist C. et al. (1981) *Nature* Vol.290:304 ff). However, in the case of proteins such as certain proteases, cytotoxic or cytostatic proteins that interfere with the cell membranes or proteins like certain receptors, whose normal biological function triggers a response to the host cell environment (media components,

temperature etc.) that is detrimental to the host cell, expression of the proteins may negatively effect the physiology of the host cell. In other cases overexpression of a desired gene may simply be unduly taxing for the producing cells. In such cases it is desirable to inhibit the expression of the desired gene until an optimal cell density has been achieved, and only then, after an optimal period of cell culture *in vitro* or cell growth and development *in vivo* (determined empirically), induce gene expression in the cells to produce sufficient quantities of the protein. While a number of systems have been proposed and tried (as generally reviewed by Yarranton, G.T. 1992 Current Opinion in Biotechnology Vol. 3:506-511) many such systems do not allow for tight repression and subsequent complete activation. Others employ impractical activation steps that are not expected to be useful in large scale fermentation or in whole animals. The current invention however fulfills all these criteria in eucaryotic expression systems using a transcriptional switch based on procaryotic control elements.

Aspects of the tightly regulatable genetic switch used in this invention for controlling gene transcription are described in Gossen & Bujard, 1992, Proc. Natl. Acad. Sci. USA 89:55475551 and in US patent application Serial No. 08/076,726, entitled "Tight Control of Gene Expression in Eucaryotic Cells by Tetracycline-responsive Promoters" filed 14 June 1993, the full contents of both of which are incorporated herein by reference.

The genetic switch employed in this invention comprises two components: (i) a polynucleotide (e.g. DNA) moiety encoding a tetracycline-controllable transcriptional activator (also referred to herein as a "transactivator" or tTA) and (ii) a gene of interest operably linked to, i.e., under the transcriptional control of, a promoter responsive to the transcriptional activator.

The tetracycline-controllable transactivator (tTA) is composed of a procaryotic tet repressor (tetR) (also referred to as the first polypeptide) operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells (also referred to as the second polypeptide). Typically, nucleotide sequences encoding the first and second polypeptides are ligated to each other in-frame to create a chimeric gene encoding a fusion protein, although the first and second polypeptides can be operably linked by other means that preserve the function of each polypeptide (e.g., chemically crosslinked). In one embodiment, the second polypeptide is a transcriptional activating protein such as the acidic transactivating domain of virion protein 16 (VP16) of herpes simplex virus (HSV) as in plasmids pUHD15-1 or pUHD151-1 (see Figure 11). It should be appreciated that other transactivators, including acidic, proline- or serine/threonine- or glutamine-rich transactivating moieties as described below, may be substituted for the VP16 transactivator in the tetracycline-controllable fusion transactivator. In this embodiment, the second polypeptide of the fusion protein is capable of directly activating transcription.

In another embodiment, the second polypeptide of the tTA fusion protein indirectly activates transcription by recruiting a transcriptional activator to interact with the tetR fusion



protein. For example, tetR can be fused to a polypeptide domain (e.g., a dimerization domain) capable of mediating a protein-protein with a transcriptional activator protein, such as an endogenous activator present in a host cell. It has been demonstrated that functional associations between DNA binding domains and transactivation domains need not be  
5 covalent (see e.g., Fields and Song (1989) *Nature* 340:245-247; Chien et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9578-9582; Gyuris et al. (1993) *Cell* 75:791-803; and Zervos, A.S. (1993) *Cell* 72:223-232). Accordingly, the second polypeptide of the tTA fusion protein may not directly activate transcription but rather may form a stable interaction with an endogenous polypeptide bearing a compatible protein-protein interaction domain and transactivation  
10 domain. Examples of suitable interaction (or dimerization) domains include leucine zippers (Landschulz et al. (1989) *Science* 243:1681-1688), helix-loop-helix domains (Murre, C. et al. (1989) *Cell* 58:537-544) and zinc finger domains (Frankel, A.D. et al. (1988) *Science* 240:70-73). Interaction of a dimerization domain present in the tTA fusion protein with an endogeneous nuclear factor results in recruitment of the transactivation domain of the nuclear  
15 factor to the tTA, and thereby to a tet operator sequence to which the tTA is bound.

A variation of this approach is to construct a fusion of the tetR DNA binding sequence to the non-DNA binding amino acid sequences of the TATA binding protein (TBP) (TBP is described in Kao, C.C. et al. (1990) *Science* 248:1646-1650). The DNA binding form of TBP is part of a protein complex designated TFIID. The function of TBP in the  
20 complex is to recruit other protein components of the TFIID complex to position near the transcription initiation site of eucaryotic genes containing a TATA box (i.e., TBP binding site). When bound to the TATA box, the TFIID complex subsequently mediates the sequential recruitment of other members of the basic transcriptional initiation complex, resulting in initiation of transcription (described in more detail in Buratowski, S. et al. (1989)  
25 *Cell* 56:549-561). Accordingly, when fused to tetR DNA binding sequences, TBP may recruit other members of the basic transcription initiation complex to DNA sequences containing a tet operator(s). Furthermore, by substituting a TATA sequence present in a eukaryotic gene of interest with a tet operator(s), the tetR/TBP fusion protein can be targeted to this site in a manner dependent on the presence or absence of Tc (or analogue thereof),  
30 resulting in Tc-dependent initiation of transcription. Since, in this approach, the gene of interest to be regulated by the tTA (i.e., tetR/TBP fusion protein) lacks a functional TATA element, the basal level of expression of the gene in the presence of Tc (or analogue) is expected to be very low. However, upon removal of Tc (or analogue), transcription initiation is restored via binding of tetR/TBP to the tet operator(s) and recruitment of other components  
35 of the transcription initiation complex.

The tTA may be expressed in a desired host cell using otherwise conventional methods and materials by transfecting or transforming the host cell with the tTA-encoding DNA operably linked to a conventional promoter (such as are mentioned elsewhere herein), e.g. for constitutive expression.

The second component of the genetic switch is the tTA-responsive transcriptional promoter to which the gene of interest is operably linked. The promoter may be a minimal promoter comprising, for example, a portion of the cytomegalovirus (CMV) IE promoter, operably linked to at least one tet operator sequence, derived for example from the tetracycline resistance operon encoded in Tn10 of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural biology" in Protein-Nucleic Acid Interaction, Saeger & Heinemann eds., Macmillan, London, 1989, Vol.10, pp.143-162), to serve as target sequences for a tTA.

Other suitable minimal promoters include PhCMV\*-1, PhCMV\*-2, and PtK\*-1, described herein, or other minimal promoters derived from promoter elements typically used in the cell line employed as described in the references throughout this application.

Minimal promoter elements particularly useful for a given cell line may be selected from a series of deletion mutants of the original promoter nucleotide sequence, based on the ability of a given member of the series (for instance, placed as a XhoI/SacII fragment into the corresponding restriction sites of plasmid pUHC13-3) to be activated in transient transfection experiments using a cell line stably expressing the tetR-VP16 fusion protein; as will be appreciated a cell line stably expressing any other fusion of tetR with a protein domain capable of activating transcription (see below) can be used. As will also be appreciated plasmid pUHC13-3 may be modified for the specific application by replacing genetic elements like poly-adenylation sites or splice sites with those functioning in the cell line in question. Specific details may be found in the references throughout this application or references cited therein, the full contents of which are incorporated herein by reference. A second criterion for the selection of the optimal minimal promoter is the degree of repression in the presence of tetracycline (see below). Typically the deletion mutant with the highest activation factor as described below is chosen.

Promoter deletion mutants may be prepared as generally described by Rosen, C. et al (1985) Cell Vol. 41, 813-823 or Nelson C. et al. (1986) Nature Vol. 322, 557-562. Other methods, including methods useful in the preparation of stable tetR-VP16 cell lines, are essentially as described in "Current Protocols in Molecular Biology" Ausubel, F.M. et al (eds.) 1989 Vol. 1 and 2 and all supplements to date Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, New York, the full contents of which are incorporated herein by reference, or as described in the other references cited throughout this application.

The presence of tet operator element(s) renders such recombinant promoter moieties responsive to the tTA of the invention. In HeLa cells constitutively expressing the TetR-VP16 tTA, high levels of luciferase expression have been achieved under the control of such a modified CMV promoter sequence. The incorporation of the tetR domain within the tTA renders this expression system sensitive to the presence of tetracycline. The binding of tetracycline to the tetR domain of the tTA prevents the tTA from exerting its transactivating

effects. Depending on the concentration of tetracycline in the culture medium (0-1  $\mu\text{g/ml}$ ), the luciferase activity can be regulated up to five orders of magnitude in the previously mentioned example. This system provides both a reversible on/off switch and a differential control--as desired--for regulating gene expression in eucaryotic hosts. It should be appreciated that tetracycline analogs which are capable of specific functional interaction with tetR may be used in place of tetracycline,

A eucaryotic production cell line of this invention is prepared according to the design described above. Assembly of the components and incorporation thereof into a eucaryotic host cell are conducted by otherwise conventional methods such as are described generally by Kriegler, M. (editor), 1990, *Gene Transfer and Expression, A Laboratory Manual* (Stockton Press). Care should be taken to select for integration of the gene of interest into a chromosomal site that exhibits sufficiently low basal expression when, or to the extent, desired (see e.g. Table 1). The recombinant host cell obtained is grown in the presence of tetracycline or tetracycline analogues until an optimal density that has been determined empirically to allow for subsequent induction of gene expression. After the desired cell density has been reached gene expression is induced by dilution and/or removal of the tetracycline or analog thereof. The culture is then continuously grown until a optimal expression level has been reached. The recombinant protein is then harvested according to standard procedures.

The use of eucaryotic cells as host cells for expression of recombinant proteins is generally reviewed in M.Kriegler 1990 "Gene Transfer and Expression, A Laboratory Manual". Stockton Press., incorporated herein as reference. While CHO<sup>dhfr</sup>- cells (Urlaub, G. and Chasin (1980) *Proc.Natl. Acad. Sci. USA* 77: 4216-4220), 293 cells (Graham, F.L. et al. (1977) *J. Gen.Virol.* 36: pp. 59) or myeloma cells like SP2 or NSO (Galfre, G. and Milstein, C. (1981) *Meth Enzymol.* 73 (B): 3-46) are commonly used it should be clear to those of ordinary skill in the art, that any eucaryotic cell line can be used in the practice of the subject invention, so long as the cell line is not incompatible with the protein to be expressed, the selection system chosen or the fermentation system employed. This invention is broadly applicable and encompasses non-mammalian eucaryotic cells as well, including insect (e.g. *Sp. frugiperda*), yeast (e.g. *S. cerevisiae*, *S. pombe*, *H. polymorpha*) and fungal cells, containing and capable of expressing the two components of the foregoing genetic switch.

The eucaryotic host cells used for regulated expression in this invention may thus be yeast cells including, but not limited to *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis* and *Hansenula polymorpha*, as generally reviewed by Fleer, R. (1992), *Current Opinion in Biotechnology* Vol. 3, No. 5: p. 486-496, the full contents thereof and of which the references cited therein are incorporated herein by reference.

In other embodiments the eucaryotic cells used for regulated expression are insect cells carrying in their chromosomes the heterologous DNA moiety encoding a transactivator fusion protein (tTA) comprising a tetracycline repressor and a protein capable of activating

transcription in the host cell. A second recombinant DNA moiety encoding the gene of interest operably linked to a promoter responsive to the transcriptional activator is carried on the baculovirus genome. Suitable general methods which may be used in the practice of these aspects of the invention are reviewed by O'Reilly et al. (1992) "Baculovirus expression  
5 vectors, A Laboratory Manual" Stockton Press, the full contents of which are incorporated herein by reference.

While the gene of interest may be a heterologous gene, i.e. not otherwise present in the parental host cell genome, an important aspect of this invention relates to the regulation of an endogenous gene of interest. In such cases the host cell is genetically engineered to insert  
10 into the host cell genome the tTA-responsive promoter such that the desired endogenous gene is under the transcriptional control of the tTA-responsive promoter. This may be accomplished for example by linking a copy of the endogenous gene to the tTA-responsive promoter and transfecting or transforming the host cell with the recombinant construct. In one approach, the construct is introduced by homologous recombination into the loci of the  
15 endogenous gene. Briefly, the tTA responsive promoter is flanked on the 5' side by sufficient DNA sequences from the upstream region but excluding the actual promoter region of the endogenous gene and on the 3' end by sequences representing the coding region of the endogenous gene. The extent of DNA sequence homology necessary for homologous recombination is discussed below.

20 In other approaches that construct is inserted at another genetic locus, either predetermined or at random. In any case, the eucaryotic cell is also transformed or transfected with the DNA construct permitting expression of the tTA. Alternatively, the DNA construct encoding the tTA may itself be inserted at the locus of the endogenous gene of interest and the DNA moiety encoding the gene of interest operably linked to a  
25 tTA-responsive promoter may be introduced elsewhere in the genome. In that embodiment, the tTA vector contains the tTA-encoding DNA moiety flanked by DNA sequence of the locus of the endogenous gene permitting homologous recombination of the construct into that locus.

The use of flanking DNA sequence to permit homologous recombination into a  
30 desired genetic locus is known in the art. At present it is preferred that up to several kilobases or more of flanking DNA corresponding to the chromosomal insertion site be present in the vector on both sides of the tTA-encoding sequence (or any other sequence of this invention to be inserted into a chromosomal location by homologous recombination) to assure precise replacement of chromosomal sequences with the exogenous DNA. See e.g.  
35 Deng et al, 1993, Mol. Cell. Biol 13(4):2134-40; Deng et al, 1992, Mol Cell Biol 12(8):3365-71; and Thomas et al, 1992, Mol Cell Biol 12(7):2919-23. It should also be noted that the eucaryotic cell of this invention may contain multiple copies of the gene of interest, e.g. by conventional genetic amplification, each operably linked to the tTA-responsive promoter.

It should be clear from the preceding that to achieve the goals of introducing the DNA moiety encoding the tTA into the host cell genome and of introducing the tTA-responsive promoter construct in operable linkage to the desired gene, vectors based on the following principles are required. First, to introduce the tTA-encoding construct into the genome of the host cell such that its expression will follow the regulated pattern of expression observed in the unmodified host cell for the gene of interest, it is necessary to introduce the tTA-encoding construct such that its expression is made subject to the transcription control elements associated with the gene of interest. One way to do so is to introduce the tTA-encoding construct by homologous recombination into the genetic locus of the gene of interest. A vector for such introduction comprises the DNA sequence encoding the tTA flanked by sufficient DNA sequence from the locus of the gene of interest in the host genome to permit the desired homologous recombination event in which the tTA and flanking DNA is effectively swapped for the flanking DNA copy and the DNA included therebetween within the host cell genome. As will be appreciated an expression construct containing a tTA responsive promoter operably linked to the DNA sequence of the endogenous gene can be integrated at random sites without the help of flanking homologous sequences as described in references throughout this application. Alternatively, to insert a DNA sequence comprising a tTA-responsive promoter or tetO control element(s) upstream of a desired gene, a construct is assembled in which the DNA comprising the tTA-responsive promoter is ligated upstream of a copy of the desired gene between DNA sequences flanking the desired insertion site in the host genome. In either event the tTA construct can be introduced as mentioned previously.

Using the foregoing genetic constructs and engineered eucaryotic cells, this invention further provides a method for regulating the expression of a gene of interest. In one aspect of this method eucaryotic host cells engineered as described above are cultured under otherwise conventional conditions suitable for cell growth and proliferation, but in a culture medium containing a substance capable of binding to the tetracycline repressor moiety and of blocking or inhibiting transcriptional activation. Tetracycline is the archetypical such substance. However, tetracycline analogs which bind to tetR to form a complex which is not transcriptionally activating may of course be substituted for tetracycline. The precise concentration of tetracycline or other such substance will depend on the substance's affinity for the tetR domain and/or the substance's specific inhibitory activity, as well as the cell density and copy number of the tTA and the desired level of inhibition of gene expression. Nonetheless, appropriate levels of inhibitory substance for the desired level of inhibition are readily determinable empirically without undue effort.

Cell culture in accordance with the preceding method negatively regulates, i.e. inhibits expression of the gene of interest, completely or partially. Culturing of the cells thereafter in media with a lower concentration (relative to the initial concentration) of the tetR binding substance permits gene expression to begin or to ensue at a now higher level. If an initial concentration of binding substance (e.g. tetracycline) is selected which is sufficient

to inhibit gene transcription substantially completely (e.g. transcription is not observed under conventional Northern blotting conditions), and in the following phase of cell culture the binding substance is substantially removed from the media, gene expression can be said to be regulated in an on/off manner. In some applications, intermediate levels of expression may be desired. To that end, concentrations of binding substance may be selected based on empirical data to provide predetermined intermediate level(s) of gene transcription. It should be understood that removal of the binding substance from the media may be effected by gradual, step-wise, continual or total replacement of culture media containing the binding substance with culture media lacking the binding substance or simply containing reduced levels of the binding substance.

Where the eucaryotic cells engineered in accordance with this invention are incorporated into the host organism, e.g. to create a transgenic organism, this invention provides a genetically engineered non-human animal capable of regulatably expressing a gene of interest. Such animal, in the broad sense, comprises cells containing and capable of expressing a heterologous DNA moiety encoding a tTA as previously defined and a DNA moiety comprising an gene of interest under the transcriptional control of a heterologous promoter responsive to the transcriptional activator.

Thus, this invention further relates to non-human animals derived by homologous recombination of one or more polynucleotide molecules of the invention into a specific target site within their genome, the offspring of such animals, as well as to a method to prevent or promote the expression of a targeted gene in a conditional manner.

This embodiment of the invention is able to solve a longstanding problem in the field generally described as gene targeting or gene knock out (Capecchi, M.R. (1989) Science Vol 244, p. 1288-1292, Bradley, A. (1991) Current opinion in Biotechnology Vol. 2, p. 823-829) pertaining to genes whose mutations results in death of the homozygous embryos, e.g., as described for the knock out of the RB gene (Jacks, T. et al. (1992) Nature 359:295-300). If the genetic switch subject of the current invention is applied as described below, expression of an endogenous gene of interest operably linked to a tet operator sequence(s) can be stimulated by a tetracycline-controllable transactivator (tTA) of the invention and the animal develops like a nonmutated wildtype animal. Then, at a particular stage of development, expression of the endogeneous gene of interest can be switched off by raising the level of tetracycline or a tetracycline analogue in the circulation and the tissues of the animal by feeding or injecting the tetracycline or tetracycline analog to the animal, thereby inhibiting the activity of the tTA and transcription of the gene of interest. This method is generally referred to herein as a "conditional knockout".

As will be clear from the following, two principally different approaches have been devised to apply the genetic switch of this invention to the genome of the non-human animal in a way, that will allow for a temporally and spatially correct expression of the endogenous gene. In one approach, the two elements of the genetic switch are in separate locations in the

chromosome and require two integration steps, another one achieves the desired result in one step.

In the first step of one embodiment of the invention non-human animals are derived by homologous recombination of the DNA sequences of the tTA into a specific DNA site  
5 containing the nucleotide sequences of an endogenous gene of interest in such a way that part or all of the coding sequence of the endogenous gene is replaced with the tTA gene. This can be accomplished (see Figure 11) in the following steps:

- 10 (1) assembling a chimeric gene in which the sequence of the first (i.e. tTA) polynucleotide molecule of the invention is flanked by DNA sequences from the gene of interest such that upon incorporation of the chimeric gene into the host genome, the DNA sequences that normally control the expression of the target gene are fused to and control expression of the DNA sequences for the tTA.
- 15 (2) introducing this chimeric gene into an embryonic stem cell line from a species of interest and screening resultant candidate embryonic cell clones to identify and recover those cells in which homologous recombination has taken place at the locus of interest.
- 20 (3) introducing those recombinant cells so identified and recovered into a blastocyst from the species of interest to yield a chimeric embryo.
- (4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth of a homologous recombinant offspring.

25 This process results in offspring whose genome contains the DNA sequence encoding the tTA inserted in place of the gene of interest such that the tTA DNA is expressed in a pattern similar or identical to that of the gene of interest. These processes and their results are collectively and commonly referred to as "gene knock-out". These techniques are well  
30 established and described in: Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. Nature Genetics 1:92-97 & Soriano et al. Cell 64:693-702 and references therein, the full content of which are in their entirety incorporated herein by reference.

The second step in this embodiment of the invention relates to the preparation of a second transgenic animal which contains in it's genome the gene of interest under  
35 transcriptional control of the tetracycline (Tc) responsive promoter element. This can be accomplished using the following method:

- (1) A chimeric DNA sequence is prepared where a Tc responsive promoter element, (comprising at least one tet operator and a minimal promoter) is cloned 5' of the DNA

sequences encoding the endogenous gene of interest. One way to accomplish this is to replace the luciferase coding sequence and all polyadenylation elements in the plasmids pUHC13-3 or pUHC13-4 with the DNA sequence containing the complete genomic coding sequence of the endogenous gene and sufficient 3' non coding sequence to allow for proper polyadenylation. As will be appreciated the DNA sequence encoding the endogenous gene can also be cDNA (cloned as an example in such a way that it replaces exactly the luciferase gene in pUHC13-3 or pUHC13-4 ) or any combination of genomic DNA and cDNA designed to provide the complete coding sequence, any regulatory elements that may reside in intron sequences or is not contained in it's entirety in the cDNA and a polyadenylation signal or other elements typically associated with the endogenous gene. General cloning and DNA manipulation methods are described in references cited throughout this application.

(2) The chimeric DNA sequence (called also "the chimeric transgene") is injected into a fertilized egg which is implanted into a pseudopregnant recipient mother and allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster et al. (1986) Proc.Natl.Acad.Sci. USA Vol. 83:9065-9069, the full contents of which are incorporated by reference herein, that about 25 % of mice which develop will inherit one or more copies of the microinjected DNA. A protocol for constructing such transgenic animals (Brinster et al. Proc. Natl. Acad. Sci. 83:4432-4445, Crenshaw et al. Genes 3: Dev 9:959-972 and references cited therein) is a well established technique as is the breeding of recombinant and hybrid animals.

Breeding of animals resulting from the first and the second step of this embodiment of the invention produces offspring containing both the replaced gene of interest and the chimeric transgene. In a preferred embodiment, animals heterozygous for the knockout of the endogenous gene resulting from the first step of this embodiment of the invention (and instead expressing a tTA gene) are used for breeding with animals that are homozygous for the chimeric transgene resulting from the second step of this embodiment of the invention. The resulting offspring are analyzed by standard techniques, including tail-blot analysis described in references throughout this application, and animals homozygous for both traits are selected. Typically about 50 % of the offspring should carry both traits. In these animals, replacement of the coding sequences of the gene of interest with those of the DNA sequences of the tTA is such that the tTA is expressed in a temporal and spatial pattern similar or identical to that of the gene of interest and regulates in *trans* expression of the gene of interest now under transcriptional control of (i.e., operably linked to) the DNA sequences of the Tet



operator and minimal promoter inserted at its 5' end.

As will be appreciated, the particular breeding strategy depends on the nature of the gene of interest. If the "knock out" of the endogenous gene with the tTA coding sequence is not lethal and the overall plan is to create animals where the functions of the gene of interest in the adult can be studied in the "on" or "off" state, the animals from the first step of this embodiment of the invention can be bred to homozygosity and then bred with the homozygous mice from the second step.

In this combination, the gene of interest is regulated by the addition or subtraction of tetracycline or its analogs from the food or water supply of the animal as discussed below.

10 In another embodiment of the invention, embryonic stem (ES) cell technology is used to prevent or promote expression of a gene of interest in a conditional manner (Figure 12). In the first step of this embodiment of the invention, a chimeric DNA sequence (commonly referred to as a chimeric transgene) consisting of the DNA sequences of the tet operator(s) and a suitable minimal promoter inserted 5' of the DNA sequences encoding a gene of interest is introduced by stable, non-homologous recombination into random sites in the ES cell genome. Co-introduced with this chimeric construct is a selectable marker that enables the selection of cell clones that have integrated DNA constructs from cells that have not. As will be appreciated, the feeder cells supporting the growth of the ES cells have to express the same resistance gene used for the selection step. As an example, if the selection marker chosen is the hygromycin resistance gene, the primary feeder layer cells used for the ES cell culture can be derived from an animal transgenic for the hygromycin resistance gene prepared according to standard procedures for the preparation of transgenic animals, as cited throughout this application. ES cell clones are selected for low basal expression of the chimeric transgene using customary detection methods, such as evaluating the mRNA levels of the transgene as described in "Current Protocols in Molecular Biology" Ausubel, F.M. et al (eds.) 1989 Vol. 1 and 2 and all supplements to date, Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, New York, the full contents of which are incorporated herein by reference, or as described in the other references cited throughout this application. Other methods to detect expression of the transgene may include activity assays or assays designed to detect protein expression. Low basal expression of the transgene is determined relative to untransfected cells. Alternatively, low basal expression of the tet operator-linked transgene can be evaluated in different tissues of animals derived from the embryonic stem cells. For example, ES cells can be transfected with the transgene in culture, and the clones expanded, selected and injected into blastocysts to create transgenic animals. After standard identification and breeding to create animals carrying the transgene in all tissues, the baseline expression of the tet-operator linked transgene can be examined in various tissues of interest (e.g., by conventional techniques for analyzing mRNA expression, such as Northern blotting, S1 nuclease mapping or reverse transcriptase-polymerase chain reaction). Additionally, basal expression of the transgene can be examined in primary

cultures of cells derived from various tissues of the animal (e.g. skin cells in culture).

A second criterion for the selection of the stable clone is the ability of the tet operator-linked transgene to respond to transient or stable expression of tTA upon transfection of a tTA expression plasmid like pUHD15-1 or pUHD151-1. As will be appreciated, these  
5 plasmids are cited as examples only and others can be devised that expressed sufficient quantities of tTA in ES cells. The ability of tTA to induce expression of a tet-operator linked transgene stably transfected into an ES cell clone can be examined by supertransfecting the ES cell clone with a tTA expression plasmid and assaying expression of the transgene. Alternatively, inducibility of a tet-operator linked transgene can be examined in cells derived  
10 from various tissues of a transgenic animal carrying the transgene by preparing primary cultures of cells from the animal (e.g., skin cell cultures), transfecting the cells with a tTA expression plasmid and assaying expression of the transgene in the cells by standard techniques.

A clone fulfilling the criteria discussed above is selected and expanded in number.  
15 This clone is then used as a recipient of a gene knock-out procedure consisting of the following steps:

(1) flanking the sequences of a polynucleotide molecule encoding a tTA of the invention by DNA sequences from a second gene of interest such that the DNA  
20 sequences that normally control the expression of the second target gene of interest are fused to and control expression of the DNA sequences of encoding the tTA;

(2) introducing this chimeric gene into an embryonic cell line from a species of interest and modified as described above and screening candidate embryonic cell  
25 clones for those in which homologous recombination has taken place at the locus of interest;

(3) introducing those recombinant cells into blastocysts from the species of interest;  
and  
30

(4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth of a homologous recombinant animal.

This process results in offspring containing a replacement of the amino acid coding  
35 sequences of the second gene of interest with those of the DNA sequences of the tTA such that the tTA encoding sequence is expressed in a temporal and spatial pattern similar to that of the endogenous second gene of interest. In this case, it is necessary to self cross the recombinant animals (or breed to homozygosity) so that both copies of the target sequence into which the tTA coding sequences have been integrated are interrupted. This procedure

also leads to homozygosity of the tet-operator linked transgene (i.e., animals homozygous for both components of the genetic switch described herein can be produced). These techniques are well established and described in: Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. Nature Genetics 1:92-97; and Soriano et al. Cell 64:693-702 and references therein.

5 In yet another embodiment of the invention, embryonic stem (ES) cell technology can again be used to prevent or promote expression of a gene interest in a conditional manner using a single homologous recombination step that will result in the integrated copy shown in Figure 13. In this method, a DNA construct containing a fusion of the sequences that normally flank the endogenous gene of interest at the 5' end (and contain sequences  
10 commonly referred to as promoter sequences) are fused to the DNA sequences encoding the tTA molecule. At the 3' end of the tTA coding sequence, DNA sequences encoding resistance to a selectable marker are typically included. For example, a neomycin resistance gene, which may be fused to either a constitutive regulatory element (e.g., a pPGK promoter as depicted in Figure 13A) or to a tet operator sequence(s) (as depicted in Figure 13B) can be  
15 inserted at the 3' end of the tTA encoding sequence. When the selectable marker is operably linked to a tet operator sequence(s), its expression is regulated by the tTA (e.g., a resistance phenotype will be expressed in the absence but not the presence of Tc). Finally, 3' of the selectable marker sequences in this DNA construct are inserted the DNA sequences encoding the endogenous gene of interest, which are also fused to at least one tet operator sequence and  
20 a minimal promoter.

Because in this configuration of the DNA molecule, conventionally called the targeting vector, the coding sequences of the tTA, the selectable marker and the endogenous gene of interest are all flanked by the sequences normally flanking the endogenous gene of interest, this DNA construct has the potential for homologous recombination with the locus  
25 of the endogenous gene of interest upon its introduction into cells such as, but not limited to ES cells. Homologous recombination of this type alters the natural locus such that the gene of interest falls under the control of the tTA and consequently under regulation by the presence or absence of tetracycline or derivative thereof. The expression of the tTA protein, on the other hand, follows the normal pattern of expression of the gene of interest.  
30 Recombinant ES cells of this type are then used to generate intact organisms as has been described (Wood et al. Proc. Natl. Acad. Sci. 90:4582-4585, Simon et al. Nature Genetics 1:92-97; and Soriano et al. Cell 64:693-702) which can in turn be bred to homozygosity.

As will be appreciated, the close proximity of the promoter elements in this particular configuration of the DNA construct used for homologous recombination may require special  
35 consideration to insulate the downstream tet operator/minimal promoter operably linked to the endogenous gene from long range effects of the endogenous promoter operably linked to the tTA coding sequence to achieve the required low basal level expression of the endogenous gene. Some possible solutions are strong transcriptional terminators known to those of ordinary skill in the art, DNA elements that increase the distance between the

elements or others that limit the effect of enhancer sequences (e.g., transcriptional insulators, including matrix attachment regions), all of which are to be cloned alone or in combination in between the selectable marker expression unit (e.g., neomycin resistance gene with linked promoter) and the tTA-responsive transcriptional promoter sequence (see Figure 13).

- 5 Examples of suitable transcriptional terminators, transcriptional insulators, matrix attachment regions and/or other sequences which can be included in the "single hit" targeting vector to inhibit basal transcription of the tet operator-linked endogenous gene are described in Sato, K. et al. (1986) *Mol. Cell. Biol.* 6:1032-1043; Michel, D. et al. (1993) *Cell. Mol. Biol. Res.* 39:131-140; Chung, J.H. et al. (1993) *Cell* 74:505-514; Neznanov, N. et al. (1993) *Mol. Cell.*  
10 *Biol.* 13:2214-2223; and Thorey, I.S. et al. (1993) *Mol. Cell. Biol.* 13:6742-6751.

The different animals resulting from any of the above mentioned embodiments can be studied either in the absence (endogenous gene switched "on") or presence (endogenous gene switched "off") of tetracycline or tetracycline analogues as described for other transgenic animals below. Such animals can be used to identify, compare and characterize the activity of  
15 substances which interact with, upon or through the action of the gene product of interest.

The present invention relates to a control system that in eucaryotic cells allows regulation of expression of an individual gene over up to five orders of magnitude. This system is based on regulatory elements of a tetracycline resistance operon, e.g. Tn10 of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp.  
20 143-162), in which transcription of resistance-mediating genes is negatively regulated by the tetracycline repressor (tetR). In the presence of tetracycline or a tetracycline analogue, tetR does not bind to its operators located within the promoter region of the operon and allows transcription. By combining tetR with a protein capable of activating transcription in  
25 eucaryotes, e.g. the C-terminal domain of VP16 from HSV (known to be essential for the transcription of the immediate early vital genes (Triezenberg et al., (1988) *Genes Dev.* 2:718-729), a hybrid transactivator is generated that stimulates minimal promoters fused to tetracycline operator (tetO) sequences. These promoters are virtually silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator  
30 (tTA) from binding to tetO sequences.

The specificity of the tetR for its operator sequence (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162) as well as the high affinity of tetracycline for tetR (Takahashi et al., *J. Mol. Biol.* 187:341-348 (1986)) and the  
35 well-studied chemical and physiological properties of tetracyclines constitute a basis for an inducible expression system in eucaryotic cells far superior to the lacR/O/IPTG system. This has already been demonstrated in plant cells, in which direct repressor action at promoter sites is efficiently reversed by the antibiotic (Gatz & Quail, (1988) *Proc. Natl. Acad. Sci. USA* 85:1394-1397, Gatz et al., (1991) *Mol. Gen. Genet.* 227:229-237). However, these

previous systems used a tet repressor alone to inhibit gene expression, which may be inefficient or require high concentrations of the repressor intracellularly to function effectively. In contrast, the tTA of the present invention functions as a transcriptional activator to stimulate expression of a tet operator-linked gene.

5 In particular, the invention relates to a polynucleotide molecule coding for a transactivator fusion protein comprising the tet repressor (tetR) and a protein capable of directly or indirectly activating transcription in eucaryotes. The portion of the polynucleotide molecule coding for tetR may be obtained according to Altschmied et al., EMBO J. 7:4011-4017 (1988), the contents of which are fully incorporated by reference herein. Other  
10 tetR sequences are available from Genbank and/or are disclosed in Waters, S.H. et al. (1983) Nucl. Acids Res. 11:6089-6105; Unger, B. et al. (1984) Gene 31:103-108, Unger, B. et al. (1984) Nucl. Acids Res. 12:7693-7703; Tovar, K. et al. (1988) Mol. Gen. Genet. 215:76-80; Hillen, W. and Schollmeier, K. (1983) Nucl. Acids Res. 11:525-539 and Postle, K. et al. (1984) Nucl. Acids Res. 12:4849-4863, the contents of each of which are fully incorporated  
15 herein by reference.

The portion of the polynucleotide molecule coding for the negatively charged C-terminal domain of HSV-16, a protein known to be essential for transactivation in eucaryotes, may be obtained according to Triezenberg et al., Genes Dev. 2:718-729 (1988), the contents of which are fully incorporated by reference herein. Preferably, the activating  
20 domain comprises the C-terminal 130 amino acids of the virion protein 16. Alternatively, other polypeptides with transcriptional activation ability in eucaryotic cells can be used in the tTA of the invention. Transcriptional activation domains found within various proteins have been grouped into categories based upon similar structural features. Types of transcriptional activation domains include acidic transcription activation domains, proline-rich transcription  
25 activation domains, serine/threonine-rich transcription activation domains and glutamine-rich transcription activation domains. Examples of acidic transcriptional activation domains include the VP16 regions already described and amino acid residues 753-881 of GAL4. Examples of proline-rich activation domains include amino acid residues 399-499 of CTF/NF1 and amino acid residues 31-76 of AP2. Examples of serine/threonine-rich  
30 transcription activation domains include amino acid residues 1-427 of ITF1 and amino acid residues 2-451 of ITF2. Examples of glutamine-rich activation domains include amino acid residues 175-269 of Oct1 and amino acid residues 132-243 of Sp1. The amino acid sequences of each of the above described regions, and of other useful transcriptional activation domains, are disclosed in Seipel, K. et al. (EMBO J. (1992) 13:4961-4968).

35 The polynucleotide molecule coding for tetR may be linked to a polynucleotide molecule coding for the activating domain (e.g., of HSV VP16) and recombined with vector DNA in accordance with conventional recombinant DNA techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid

undesirable joining, and ligation with appropriate ligases. Alternatively, nucleic acid fragments encoding the repressor and the activating domain can be obtained by polymerase chain reaction amplification of appropriate nucleotide sequences using template DNA encoding either the repressor or the activating domain (e.g., encoding VP16). The amplified DNA fragments can then be ligated such that the protein coding sequences remain in-frame and the chimeric gene so produced can be cloned into a suitable expression vector.

Preferably, the polynucleotide molecule coding for the transactivator fusion protein further comprises an operably linked promoter. The promoter may be an inducible promoter or a constitutive promoter. Examples of such promoters include the human cytomegalovirus promoter IE as taught by Boshart et al., *Cell* 41:521-530 (1985), ubiquitously expressing promoters such as HSV-Tk (McKnight et al., *Cell* 37:253-262 (1984)) and  $\beta$ -actin promoters (e.g. the human  $\beta$ -actin promoter as described by Ng et al., *Mol. Cell. Biol.* 5:2720-2732 (1985)), as well as promoters in combination with control regions allowing integration site independent expression of the transgene (Grosveld et al., *Cell* 51:975-985 (1987)), as well as tissue specific promoters such as albumin (liver specific, Pinkert et al., *Genes Dev.* 1:268-277 (1987)), lymphoid specific promoters (Calame and Eaton, *Adv. Immunol.* 43:235-275 (1988)), in particular promoters of T-cell receptors (Winoto and Baltimore, *EMBO J.* 8:729-733 (1989)) and immunoglobulins; Banerji et al., *Cell* 33:729-740 (1983); Queen and Baltimore, *ibid.* 741-748), neuron specific promoters (e.g. the neurofilament promoter; Byrne and Ruddle, *Proc. Natl. Acad. Sci. USA* 86:5473-5477 (1989)), pancreas specific promoters (Edlund et al., *Science* 230:912-916 (1985)) or mammary gland specific promoters (milk whey promoter, U.S. Patent No. 4,873,316 and European Application Publication No. 264,166) as well as developmentally regulated promoters such as the muring hox promoters (Kessel and Cruss, *Science* 249:374-379 (1990)) or the  $\alpha$ -fetoprotein promoter (Campes and Tilghman, *Genes Dev.* 3:537-546 (1989)), the contents of each of which are fully incorporated by reference herein. Preferably, the promoter is constitutive in the respective cell types. In one embodiment of the invention, the polynucleotide molecule encoding the transactivator is integrated at a predetermined location within a second target DNA molecule (e.g., a gene of interest within a chromosome) such that the tTA-coding sequences are placed under the control of endogeneous regulatory elements (e.g., a 5' regulatory region of a target gene of interest into which the tTA-coding sequence is integrated). Depending upon which gene the tTA-coding sequences are integrated into, the endogenous regulatory elements may provide constitutive expression of the tTA in many cell types or may limit expression of the tTA to a particular cell or tissue type.

The invention also relates to another polynucleotide molecule coding for a protein, wherein said polynucleotide is operably linked to a tTA-responsive promoter. Typically, this tTA-responsive promoter comprises a minimal promoter operatively linked to at least one tet operator (tetO) sequence. The tetO sequence may be obtained, for example, according to Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid*

Interaction, Saeger & Heinemann. eds., Macmillan. London, 1989, Vol. 10, pp. 143-162, the contents of which are fully incorporated by reference herein. Other tetO sequences which may be used in the practice of the invention may be obtained from Genbank and/or are disclosed in Waters, S.H. et al. (1983) Nucl. Acids Res. 11:6089-6105; Hillen, W. and Schollmeier, K. (1983) Nucl. Acids Res. 11:525-539; Stüber, D. and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78:167-171; Unger, B. et al. (1984) Nucl Acids Res. 12:7693-7703; and Tovar, K. et al. (1988) Mol. Gen. Genet. 215:76-80, which are fully incorporated by reference herein in their entirety. One, two, three, four, five, six, seven, eight, nine or ten or more copies of the tet operator sequence may be employed, with a greater number of such sequences allowing an enhanced range of regulation. As shown in the Examples, multiple copies of the tet operator sequence provides a synergistic effect on the ability to control expression of the heterologous protein.

The polynucleotide sequence specifying the cytomegalovirus promotor may be obtained according to Boshart et al., Cell 41:521-530 (1985), the contents of which are fully incorporated by reference herein. Preferably, positions +75 to -53 to +75 to -31 of the promotor-enhancer are employed as a minimal promotor. The promotor may be followed by a polylinker and then by the gene coding for the protein of interest. While the luciferase gene or other reporter gene, e.g. the gene coding for chloramphenicol acetyltransferase or  $\beta$ -galactosidase, may be used to demonstrate the operability of the regulatory system, the invention is not intended to be so limited. Examples of such genes include, but are not limited to the estrogen receptor, the GABA receptor, the progesterone receptor and the X-protein of HBV.

The present invention also relates to eucaryotic cells transfected with the polynucleotide molecules of the present invention. In particular, the invention relates to eucaryotic cells transfected with

(a) a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes; and

30

(b) a second polynucleotide molecule coding for a protein, wherein said second polynucleotide molecule is operably linked to a minimal promotor and at least one tet operator sequence.

The two polynucleotide molecules may reside on the same or separate vectors. In a preferred embodiment, the first polynucleotide is integrated into the chromosome of a eucaryotic cell or transgenic animal and the second polynucleotide is introduced as part of a vector. Integration may be achieved where there is crossover at regions of homology shared between the incoming polynucleotide molecule and the particular genome.

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The expression of the heterologous protein from such transfected eucaryotic cells may be tightly regulated. Unexpectedly, it has been determined that the expression system of the present invention may be used to regulate expression by about 5 orders of magnitude. In addition, it has been discovered that the expression system of the present invention allows one to rapidly turn "on" and "off" the expression of the heterologous gene in a reversible way. Moreover, it has been discovered that the expression system of the invention allows one to achieve a desired level of expression according to how much tetracycline or tetracycline analogue is employed (see Figure 3). Thus, the expression system of the present invention is a great advance in the art.

The invention also relates to transgenic animals comprising at least a first polynucleotide molecule of the present invention encoding a tTA. Such transgenic animals may be obtained, for example, by injecting the polynucleotide into a fertilized egg which is allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442 (1985), the contents of which are fully incorporated by reference herein, that about 25% of mice which develop will inherit one or more copies of the microinjected DNA. It is also possible to prepare a polynucleotide molecule comprising a milk protein promotor and microinject the DNA into the fertilized egg to give, upon development, a transgenic mammal which is capable of producing the heterologous protein in its milk, when in the absence of tetracycline or a tetracycline analog. See International Application Publication No. WO 88/00239 and European Application Publication No. O264,166, the contents of which are fully incorporated by reference herein.

The invention also relates to non-human animals and their offspring derived by homologous recombination of the DNA sequences of the first polynucleotide molecules of the invention into a specific DNA site containing the nucleotide sequences of a gene referred to as the target gene. This would be accomplished in the following steps: 1) flanking the sequences of the first polynucleotide molecule of the invention encoding a tTA by DNA sequences from the target site such that the DNA sequences that normally control the expression of the target gene are fused to and control the expression of the DNA sequences of the first polynucleotide molecules of the invention, 2) introducing this chimeric gene into an embryonic cell line from the species of interest and screening candidate embryonic cell clones for those in which homologous recombination has taken place at the target gene locus, 3) introducing those recombinant cells into a blastocysts from the species of interest, 4) implanting the chimeric embryo into the uteri of pseudopregnant recipient mothers to facilitate development and birth. This process will result in offspring containing a replacement of the amino acid coding sequences of the target gene with those of the DNA sequences of the first polynucleotide molecule of the invention such that this corresponding



amino acid sequence will be expressed in a pattern similar to that of the target gene. These processes and their results are collectively and commonly referred to as "gene knock-out". These techniques are well established and described in: Wood et al. *Proc. Natl. Acad. Sci.* 90:4582-4585, Simon et al. *Nature Genetics* 1:92-97 & Soriano et al. *Cell* 64:693-702 and references therein.

The invention also relates to a method to prevent or promote the expression of the target gene in a conditional manner. This may be accomplished by breeding an animal containing the target gene knock-out (as outlined in the preceding paragraph) with a transgenic animal derived by the following method. The transgenic animal would be constructed by inserting, by micro-injection, a chimeric DNA sequence (commonly referred to as a chimeric transgene) consisting of the DNA sequences of the second polynucleotide molecule of the invention inserted 5' of the DNA sequences encoding the amino acid sequence of the target gene into the genome of a fertilized egg which is allowed to develop into an adult animal. The protocol for the construction of such transgenic animals is a well established technique (Brinster et al. *Proc. Natl. Acad. Sci.* 83:4432-4445, Crenshaw et al. *Genes & Dev* 3:959-972 and references therein) as is the breeding of animals. From the breeding will result offspring containing both the gene knock-out and the chimeric transgene. That is, replacement of the amino acid coding sequences of the target gene with those of the DNA sequences of the first polynucleotide molecule of the invention such that this corresponding amino acid sequence will be expressed in a pattern similar to that of the target gene and, the DNA sequences of the second polynucleotide molecule of the invention inserted 5' of the DNA sequences encoding the amino acid sequence of the target gene. In this combination the target gene can be regulated by the addition or subtraction of tetracycline or its analogs from the food or water supply of the animal.

Thus, the invention also relates to a method to down regulate the expression of a protein coded for by a polynucleotide, comprising cultivating the transfected eucaryotic cells of the present invention in a medium comprising tetracycline or a tetracycline analogue. As described in the Examples, it is possible to closely control the extent of expression by carefully controlling the concentration of tetracycline or tetracycline analogue in the culture media. As shown in Figure 3, panel A, as little as 0.0001  $\mu\text{g/ml}$  of tetracycline will begin to result in a decrease of polypeptide (luciferase) expression. At about 0.1  $\mu\text{g/ml}$ , the expression is essentially shut off. The concentration of tetracycline or tetracycline analogue which can be used to regulate the expression level may range from about 0.001 to about 1  $\mu\text{g/ml}$ .

The invention also relates to a method to up regulate the expression of a protein coded for by a polynucleotide, comprising cultivating the eucaryotic cells of the present invention in a medium lacking tetracycline or a tetracycline analogue.

The invention also relates to a method to use regulated gene expression in the production of recombinant proteins as generally reviewed by Yarranton, G.T 1992, the whole article incorporated as reference herein. Expression of recombinant proteins that are cytotoxic

or otherwise interfere with physiological processes in cells has been hampered by the lack of suitable methods to tightly regulate gene expression. In contrast, a production cell line according to the current invention is grown in the presence of tetracycline or tetracycline analogues until an optimal density (assessed empirically to allow for subsequent induction of gene expression) and expression is induced by dilution of the regulating compound. The culture is continuously grown until an optimal expression level has been reached. The recombinant protein is then harvested according to standard procedures.

As a preferred embodiment, eucaryotic cells are used for expression of recombinant proteins as generally reviewed in "Gene Transfer and Expression" (M. Kriegler 1990) incorporated herein as reference. While CHO<sup>dhfr</sup>- cells (Urlaub, G. and Chasin, L. 1980), 293 cells (Graham, F.L. et al. 1977) or myeloma cells like SP2 or NS0 (Galfre, C. and Milstein, C. 1981) are commonly used it should be clear to the skilled in the art, that any eucaryotic cell line can be used that is suitable for the protein to be expressed, the selection system chosen and the fermentation system employed.

In another preferred embodiment, the cells used for regulated expression are yeast cells including, but not limited to *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis* and *Hansenula polymorpha* as generally reviewed by Flier, R. 1992, the whole article incorporated as referenced herein.

In another preferred embodiment, the cells used for regulated expression are insect cells with the gene and promoter region carried on the baculovirus genome as generally reviewed in "Baculovirus expression vectors" (O'Reilly et al. 1992), the whole document incorporated as referenced herein.

As can be appreciated, the tissue specificity of some promoters dictate that the tet operator sequence/promoter sequence fusion has to be designed with the particular application and cell line in mind following the teachings in this application using the promoters customarily used for the cell line in question; examples for those promoters are given in the relevant references mentioned above.

It should be clear from the foregoing that it is critical in the current invention that the production cell line is selected for a very low basal expression of the gene under control of the Tet operator/CMV promoter sequence. There are numerous methods currently available employing enzymatically assisted or unassisted homologous recombination to target repeatedly a chromosomal location found empirically to be suited for the integration of the gene encoding the recombinant protein. In addition to the homologous recombination approaches already described herein, enzyme-assisted site-specific integration systems are known in the art and can be applied to the components of the regulatory system of the invention to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include the Cre recombinase-lox target system (e.g., as described in Baubonis, W. and Sauer, B. (1993) Nucl. Acids Res. 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) Proc. Natl. Acad. Sci. USA 89:7905-

7909) and the FLP recombinase-FRT target system (e.g., as described in Dang, D.T. and Perrimon, N. (1992) Dev. Genet. 13:367-375; and Fiering, S. et al. (1993) Proc. Natl. Acad. Sci. USA 90:8469-8473).

Media which may be used in the practice of the invention include any media which  
5 are compatible with the transfected eucaryotic cells of the present invention. Such media are commercially available (e.g. from Gibco/BRL).

Alternatively, it is possible to down regulate the expression of a protein in a transgenic animal of the present invention by administering to the animal tetracycline or tetracycline analogue. The tetracycline or tetracycline may be administered by any means that  
10 achieves its intended purpose, e.g. by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route (see e.g., Example 2). The dosage administered will be dependent upon the age, health, and weight of the animal, kind of concurrent treatment, if any, and frequency of treatment. To up regulate the expression of the protein, the administration of  
15 tetracycline or tetracycline analogue may then be interrupted.

The invention also relates to a kit comprising a carrier means having in close confinement therein at least two container means such as tubes, vials, bottles and the like, each of which containing a polynucleotide molecule which can be used in the practice of the invention. In particular, the invention relates to a kit comprising a carrier means having in  
20 close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes in a form suitable for homologous recombination; and a second container means contains a second polynucleotide molecule comprising a minimal promotor operably linked to at least  
25 one tet operator sequence, wherein the second polynucleotide molecule is capable of being ligated to a heterologous gene sequence coding for a polypeptide and activating the expression of the heterologous protein.

The invention also relates to kits comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a  
30 eucaryotic cell transfected with a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic tet repressor and a protein capable of activation transcription in eucaryotes in a form suitable for homologous recombination; and a second container means contains a second polynucleotide molecule comprising a minimal promotor operably linked to at least one tet operator sequence, wherein the second polynucleotide  
35 molecule is capable of being ligated to a heterologous gene sequence coding for a polypeptide and activating expression of the polypeptide.

The invention is widely applicable to a variety of situations where it is desirable to be able to turn gene expression "on" and "off", or regulate the level of gene expression, in a rapid, efficient and controlled manner without causing pleiotropic effects or cytotoxicity.

The invention may be particularly useful for gene therapy purposes in humans. in treatments for either genetic or acquired diseases. The general approach of gene therapy involves the introduction of one or more nucleic acid molecules into cells such that one or more gene products encoded by the introduced genetic material are produced in the cells to restore or enhance a functional activity. For reviews on gene therapy approaches see Anderson, W.F. (1992) *Science* 256:808-813; Miller, A.D. (1992) *Nature* 357:455-460; Friedmann, T. (1989) *Science* 244:1275-1281; and Cournoyer, D., et al. (1990) *Curr. Opin. Biotech.* 1:196-208. However, current gene therapy vectors typically utilize constitutive regulatory elements which are responsive to endogenous transcriptions factors. These vector systems do not allow for the ability to modulate the level of gene expression in a subject. In contrast, the regulatory system of the invention provides this ability.

To use the system of the invention for gene therapy purposes, at least one DNA molecule is introduced into cells of a subject in need of gene therapy (e.g., a human subject suffering from a genetic or acquired disease) to modify the cells. The cells are modified to contain 1) nucleic acid encoding a tTA of the invention in a form suitable for expression of the tTA in the host cells and 2) a gene of interest (e.g., for therapeutic purposes) operatively linked to a tTA-responsive promoter (e.g., a *tet* operator sequence(s) and minimal promoter). Preferably, one or both of these DNA molecules is integrated into a predetermined location within a chromosome of the human cells by homologous recombination. A single DNA molecule encoding both components of the regulatory system of the invention can be used, or alternatively, separate DNA molecules encoding each component can be used. The cells of the subject can be modified *ex vivo* and then introduced into the subject or the cells can be directly modified *in vivo* by conventional techniques for introducing nucleic acid into cells. Expression of the gene of interest in the cells of the subject is stimulated in the absence of Tc or a Tc analogue, whereas expression is then inhibited by administering Tc or a Tc analogue to the patient. The level of gene expression can be varied depending upon which particular Tc analogue is used as the inducing agent. Additionally, expression of the gene of interest can be adjusted according to the medical needs of the individual, which may vary throughout the lifetime of the individual. Thus, the regulatory system of the invention offers the advantage over constitutive regulatory systems of allowing for modulation of the level of gene expression depending upon the requirements of the therapeutic situation.

Genes of particular interest to be expressed in cells of a subject for treatment of genetic or acquired diseases include those encoding adenosine deaminase, Factor VIII, Factor IX, dystrophin,  $\beta$ -globin, LDL receptor, CFTR, insulin, erythropoietin, anti-angiogenesis factors, growth hormone, glucocerebrosidase,  $\beta$ -glucuronidase,  $\alpha$ 1-antitrypsin, phenylalanine hydroxylase, tyrosine hydroxylase, ornithine transcarbamylase, arginosuccinate synthetase, UDP-glucuronosyl transferase, apoA1, MDR1 and MRP multidrug resistance genes, TNF, soluble TNF receptor, interleukins (e.g., IL-2), interferons (e.g.,  $\alpha$ - or  $\gamma$ -IFN) and other cytokines and growth factors.

Gene therapy applications of particular interest in cancer treatment include overexpression of a cytokine gene (e.g., TNF- $\alpha$ ) in tumor infiltrating lymphocytes or ectopic expression of cytokines in tumor cells to induce an anti-tumor immune response at the tumor site), expression of an enzyme in tumor cells which can convert a non-toxic agent into a toxic agent, expression of tumor specific antigens to induce an anti-tumor immune response, expression of tumor suppressor genes (e.g., p53 or Rb) in tumor cells, expression of a multidrug resistance gene (e.g., MDR1 and/or MRP) in bone marrow cells to protect them from the toxicity of chemotherapy.

Gene therapy applications of particular interest in treatment of viral diseases include expression of trans-dominant negative viral transactivation proteins, such as trans-dominant negative tat and rev mutants for HIV or trans-dominant ICp4 mutants for HSV (see e.g., Balboni, P.G. et al. (1993) *J. Med. Virol.* 41:289-295; Liem, S.E. et al. (1993) *Hum. Gene Ther.* 4:625-634; Malim, M.H. et al. (1992) *J. Exp. Med.* 176:1197-1201; Daly, T.J. et al. (1993) *Biochemistry* 32:8945-8954; and Smith, C.A. et al. (1992) *Virology* 191:581-588), expression of trans-dominant negative envelope proteins, such as env mutants for HIV (see e.g., Steffy, K.R. et al. (1993) *J. Virol.* 67:1854-1859), intracellular expression of antibodies, or fragments thereof, directed to viral products ("internal immunization", see e.g., Marasco, W.A. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893) and expression of soluble viral receptors, such as soluble CD4.

The regulatory system of the invention can also be used to express a suicide gene (such as a ricin or HSV tk gene) in cells in a conditional manner to allow for destruction of the cells (e.g., *in vivo*) following a particular therapy. For example, a suicide gene can be introduced into tumor cells to be used for anti-cancer immunization or into the viral genome of a live attenuated viral to be used as a vaccine. The tumor cells or viral vaccine carrying the suicide gene are administered to a subject in the presence of Tc (or analogue thereof). Following immunization, the drug is withdrawn (e.g., administration is stopped), thereby inducing expression of the suicide gene to destroy the tumor cells or cells carrying the live virus.

Cells types which can be modified for gene therapy purposes include hematopoietic stem cells, myoblasts, hepatocytes, lymphocytes, airway epithelium and skin epithelium. For further descriptions of cell types, genes and methods for gene therapy see e.g., Wilson, J.M et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano, D. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Wolff, J.A. et al. (1990) *Science* 247:1465-1468; Chowdhury, J.R. et al. (1991) *Science* 254:1802-1805; Ferry, N. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Wilson, J.M. et al. (1992) *J. Biol. Chem.* 267:963-967; Quantin, B. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Dai, Y. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; van Beusechem, V.W. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Rosenfeld, M.A. et al. (1992) *Cell* 68:143-155; Kay, M.A. et al. (1992) *Human Gene Therapy* 3:641-647; Cristiano, R.J. et al. (1993) *Proc. Natl. Acad. Sci. USA*

90:2122-2126; Hwu, P. et al. (1993) *J. Immunol.* 150:4104-4115; and Herz, J. and Gerard, R.D. (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816.

The regulatory system of the invention can also be used to produce and isolate a gene product (e.g., protein) of interest. Large scale production of a protein of interest can be accomplished using cultured cells *in vitro* which have been modified to contain 1) nucleic acid encoding a tTA of the invention in a form suitable for expression of the tTA in the host cells and 2) a gene of interest (e.g., encoding a protein of interest) operatively linked to a tTA-responsive promoter (e.g., a *tet* operator sequence(s) and minimal promoter). For example, mammalian, yeast or fungal cells can be modified to contain these nucleic acid components as described herein. Alternatively, an insect cell/baculovirus expression system can be used. To produce and isolate a gene product of interest, a host cell (e.g., mammalian, yeast or fungal cell) carrying the two components of the regulatory system of the invention (e.g., nucleic acid encoding a tTA and a gene of interest, encoding the gene product of interest, linked to a tTA-responsive promoter) are first grown in a culture medium in the presence of tetracycline or a tetracycline analogue. Under these conditions, expression of the gene of interest is repressed. Next, the concentration of tetracycline or the tetracycline analogue in the culture medium is reduced to stimulate transcription of the gene of interest. The cells are then further cultured in the absence of Tc (or analogue thereof) until a desired amount of the gene product encoded by the gene of interest is produced by the cells. The gene product can then be isolated from harvested cells or from the culture medium by standard techniques.

The invention also provides for large scale production of a protein of interest in animals, such as in transgenic farm animals. Advances in transgenic technology have made it possible to produce transgenic livestock, such as cattle, goats, pigs and sheep (reviewed in Wall, R.J. et al. (1992) *J. Cell. Biochem.* 49:113-120; and Clark, A.J. et al. (1987) *Trends in Biotechnology* 5:20-24). Accordingly, transgenic livestock carrying in their genome the components of the regulatory system of the invention can be constructed. Thus, by appropriate mating, double transgenic animals carrying a transgene encoding a tTA of the invention and a transgene comprising a tTA-responsive promoter linked to a gene of interest (the gene of interest may be either an exogenous or an endogenous gene) can be obtained. In the absence of Tc (or analogue), expression of the gene of interest is stimulated in the transgenic animals. By administering Tc (or analogue) to the animal, expression of the gene of interest can be inhibited. Protein production can be targeted to a particular tissue by linking the nucleic acid encoding the tTA to an appropriate tissue-specific regulatory element(s) which limits expression of the transactivator to certain cells. For example, a mammary gland-specific regulatory element, such as the milk whey promoter (U.S. Patent No. 4,873,316 and European Application Publication No. 264,166), can be linked to the tTA-encoding transgene to limit expression of the transactivator to mammary tissue. Thus, in the absence of Tc (or analogue), the protein of interest will be produced in the mammary tissue of

the transgenic animal, whereas protein expression can be downmodulated by administering Tc or a Tc analogue. The protein can be designed to be secreted into the milk of the transgenic animal, and if desired, the protein can then be isolated from the milk.

Having now generally described this invention, the same will be understood by reference to the following examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified. The contents of all publications, references, patents and published patent applications cited throughout the application are hereby incorporated by reference.

## 10 **Example 1: Regulation of Gene Expression in Cells by tTA**

### Materials and Methods

Construction of the transactivators tTA and tTA<sub>S</sub>. The tetR sequence was originally recovered from pWH510 (Altschmied et al., EMBO J. 7:4011-4017 (1988), the disclosure of which is fully incorporated by reference herein) by PCR and inserted into pUHD10-1 (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)), resulting in pUHD14-1 (see, the Dissertation of Manfred Gossen, "Prokaryotic Repressor Operator Systems in the Control of Eucaryotic Gene Expression, Heidelberg University, 1993, the contents of which are fully incorporated by reference herein). A unique AflII cleavage site, overlapping the tetR stop codon in this plasmid construct, allows for the in-frame insertion of coding sequences. To generate tTA, a 397-base-pair (bp) MluI/FokI fragment of pMSVP16 (Triezenberg et al., Genes Dev. 2:718-729 (1988), the disclosure of which is fully incorporated by reference herein), coding for the C-terminal 130 amino acids of VP16 of HSV, was blunted by filling in the protruding ends with T4 DNA polymerase. This DNA was inserted in pUHD14-1, previously cleaved with AflII, and blunted by mung bean nuclease. The resulting plasmid, pUHD15-1, encodes the tTA sequence (Fig. 1, panel a) under the control of the P<sub>h</sub>CMV (human cytomegalovirus promoter IE; see below). In a homologous approach, a DNA fragment coding for the 97-amino acid C-terminal portion of VP16 was fused to tetR by PCR-mediated cloning. The resulting plasmid, pUHD151-1, encodes the smaller version of the trans-activator, tTA<sub>S</sub> (Fig. 1, panel a).

### Construction of P<sub>h</sub>CMV\* and the Luciferase Reporter Plasmid.

Plasmid pUHC13-1 is a derivative of pUHD10-1 (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)). It contains the promoter-enhancer sequence of P<sub>h</sub>CMV, spanning position +75 to position -675 (Boshart et al., Cell 41:521-530 (1985)). This promoter is followed by a polylinker and the luciferase gene of Photinus pyralis fused to the SV40 small-t intron and poly(A) signal. The latter elements and the luciferase gene were transferred from pSV2L, AΔ5' (DeWit et al., Mol. Cell. Biol. 7:725-737 (1987)). By this transfer, the N-terminus of luciferase has been modified as described (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5404 (1989)). The enhancer region of P<sub>h</sub>CMV was removed

by PCR-mediated cloning, whereby a Xho I site was introduced adjacent to position -53. The resulting minimal promoter, P<sub>hCMV</sub>\* (Fig. 1, panel b) is part of the reporter plasmid pUHC13-2.

5     Construction of P<sub>hCMV</sub>\*-1 and P<sub>hCMV</sub>\*-2.

To combine P<sub>hCMV</sub>\* with tet operators, the 19-bp inverted repeat sequence of operator O2 of Tn10 (Triezenberg et al., Genes Dev. 2:718-729 (1988)) was synthesized as part of a 42-bp DNA fragment [SEQ ID NO: 10]:

(upper strand: 5' TCGAGTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAAG 3').

10    Upon annealing, the two complementary strands exposed the compatible protruding ends of a Xho I and a Sal I cleavage site at the 5 and 3' ends, respectively. Ligation of this fragment into the Xho I site of the polylinker of pT81-luc (Nordeen, S.K., BioTechniques 6:454-457 (1988)) created, upon cloning, single as well as multiple inserts of operator sequences upstream of a thymidine kinase (tk) minimal promoter from HSV contained in pT81 -luc. The  
15    tk promoters containing one, two, and seven operator sequences were examined for their ability to be activated in transient expression experiments using the HeLa cell line HtTa-1 (see below). All constructs were active in tTA producing cells in a tetracycline-dependent manner. The heptameric version of the tetO sequences caused by far the highest activation of all P<sub>tk</sub>-tetO constructs. It therefore was removed as a XhoI/SalI fragment and transferred into  
20    pUHC13-2. Due to the asymmetric location of the tetO within the polylinker of pT81-luc, the resulting plasmids pUHC13-3 and pUHC13-4 contain the heptameric tetOs in two orientations differing in the distance between the operators and position + 1 of P<sub>hCMV</sub> by 19 bp. The two tetO-containing promoters were designated P<sub>hCMV</sub>\*-1 and P<sub>hCMV</sub>\*-2 (Fig. 1, panel b).

25

Band-Shift Assay.

Cytoplasmic and nuclear cell extracts from ~ 2 x 10<sup>6</sup> cells were prepared as described by Andrews and Faller, Nucl. Acids Res. 19:2499 (1991), except that the cytoplasmic protein fraction was centrifuged once more (1 hr, 100,000 x g). Nuclear proteins were extracted by a  
30    buffer containing 20 mM Hepes-KOH (pH 7.9), 25 % glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Aliquots (5 µl) of nuclear extracts were mixed with 15 µl of binding buffer (10 mM Tris HCl, pH 7.5/10 mM MgCl<sub>2</sub>) containing 20 µg of calf thymus DNA, 5 µg of bovine serum albumin, and 2 fmol of <sup>32</sup>P-labeled tetO DNA. The tetO DNA was isolated from pUHC13-3  
35    as a 42-bp Taq I fragment whose protruding ends were filled in by Klenow enzyme in the presence of [α-<sup>32</sup>P]dCTP. After 20 min. at room temperature, aliquots of the binding reaction mixture were loaded onto a 5 % polyacrylamide/0.07 % bisacrylamide gel. Electrophoresis was carried out in 90 mM Tris base/90 mM boric acid/3 mM EDTA at 5 V/cm.



### Luciferase Assays.

Cell grown to ~ 80 % confluency in 35-mm dishes in Eagle's minimum essential medium were washed with 2 ml of phosphate-buffered saline before they were lysed in 25 mM Tris phosphate, pH 7.8/2 mM dithiothreitol/2 mM diaminocyclohexanetetraacetic acid/10 % glycerol/1 % Triton X-100 for 10 min at room temperature. The lysate was scraped off the culture dishes and centrifuged for 10 sec in an Eppendorf centrifuge. Next, aliquots (10  $\mu$ l) of the supernatant were mixed with 250  $\mu$ l of 25 mM glycylglycine/15 mM MgSO<sub>4</sub>/5 mM ATP and assayed for luciferase activity in a Lumat LB9501 (Berthold, Wildbad, F.R.G.) using the integral mode (10 sec). D-Luciferin (L6882, Sigma) was used at 0.5 mM. The background signal measured in extracts of HeLa cells that did not contain a luciferase gene was indistinguishable from the instrumental background [80-120 relative light units (rlu)/10 sec). Protein content of the lysates was determined according to Bradford (Bradford, M.M., Anal. Biochem. 72:248-254 (1976)).

## RESULTS

### Construction and Characterization of the tTA.

To convert the prokaryotic tet repressor into a eucaryotic transactivator, it was fused to the negatively charged C-terminal domain of HSV-VP16, known to be essential for transactivation. (Triezenberg et al., Genes Dev. 2:718-729 (1988)). Sequences coding for either a 97- or a 127-amino acid C-terminal portion of VP16 were fused to the tetR gene, resulting in the coding sequences of tTAS and tTA, respectively (Fig. 1, panel a). In plasmids coding for tTA (pUHD15-tTAs (pUHD 151 -1), the transactivator sequences are flanked upstream by P<sub>h</sub>CMV and downstream by the SV40 poly(A) site. The two fusion proteins did not differ in their functional *in vivo* properties.

HeLa cells transiently transfected with pUHD15-1 produced a fusion protein of the expected molecular mass (37 kDa), as demonstrated in immunoblots of the electrophoretically separated cytoplasmic and nuclear extracts (Fig. 2, panel a). When nuclear extracts were mixed with the tetO DNA, the electrophoretic mobility of the DNA was diminished. The specificity of the interaction between tTA and operator DNA was confirmed by the finding that no mobility change for tetO DNA was detectable in the presence of the specific inducer tetracycline (Fig 2, panel b).

### Construction of a tTA-Dependent Promoter.

To generate promoters activatable by tTA, tetOs were inserted upstream of minimal promoter sequences. For P<sub>h</sub>CMV, the upstream enhancer region was removed by PCR and a Xho I cleavage site was introduced adjacent to position -53. This minimal promoter, designated P<sub>h</sub>CMV\*, spans the original P<sub>h</sub>CMV sequence from +75 to -53 (+ 1 being the first nucleotide transcribed) and, in addition, contains a Stu I site around -31 (Fig. 1, panel b).

tetO sequences were fused to this core promoter by insertions at the Xho I site (Fig. 1).

The tetO sequence 02 of Tn10 is a 19-bp inverted repeat to which tetR binds as a 46-kDa dimer (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in Protein-Nucleic Acid Interaction, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162). It was chemically synthesized and ligated into the Xho I cleavage site of the polylinker located upstream of the minimal tk promoter in plasmid pT81-luc (Nordeen, S.K., BioTechniques 6:454-457 (1988)). Multiple insertions of tetOs created a set of promoters that contained between 1 and 7 tetO sequences upstream from position -81 of the tk promoter. A Xho I/Sal I fragment containing 7 tetOs, fused head to tail, was recovered from one of the constructs and transferred into the Xho I site upstream of P<sub>hCMV</sub>\*. Due to the asymmetry of the Xho I/Sal I fragment, two P<sub>hCMV</sub>\*-tetO constructs were obtained that differ in the distance between the operators and position +1 of P<sub>hCMV</sub>, which is 95 bp for P<sub>hCMV</sub>\*-1 and 76 bp for P<sub>hCMV</sub>\*-2. The plasmids containing these promoters are designated pUHC13-3 and pUHC13-4, respectively (Fig. 1, panel b). When HeLa cells were transiently transfected with these plasmids, high levels of luciferase activity were monitored whenever the cells were cotransfected with pUHD15-1, which provided the coding sequence of tTA. Little activity was observed with cultures grown in the presence of tetracycline (1.0 µg/ml) or with plasmids containing P<sub>hCMV</sub>\* only. Since P<sub>hCMV</sub>\*-1 and P<sub>hCMV</sub>\*-2 were activated by tTA to a significantly higher degree than any of the P<sub>tk</sub> constructs, the latter ones were not investigated further.

#### Quantitation of P<sub>hCMV</sub>\*-1 and P<sub>hCMV</sub>\*-2 Activation by tTA.

To quantify the stimulation of P<sub>hCMV</sub>\*-tetO constructs by tTA, HeLa cell lines were established that contained the P<sub>hCMV</sub>\*-1- or the P<sub>hCMV</sub>\*-2-luciferase, as well as the P<sub>hCMV</sub>-tTA expression units stably integrated. Conditions for culturing and selecting cells have been described (Deuschle et al., Proc. Natl. Acad. Sci. USA 86:5400-5405 (1989)). In a first step, cells were cotransfected with pUHD15-1 and pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1:327-341 (1982)). Clones resistant to G418 were assayed for transactivation of P<sub>hCMV</sub>\*-1 by transient transfection with pUHC13-3. In all HeLa cell clones in which the tetracycline-responsive promoters were active, tTA was not detectable by Western blots or by immunofluorescence. Its presence was just barely visible in electrophoretic mobility shift experiments of highly labeled tetO DNA. This indicates very low intracellular concentrations of tTA and may reflect a selection against squelching effects caused by higher concentrations of VP16-activating domains (Gill & Ptashne, Nature (London) 334:721-724 (1988)).

One of the positive clones, HtTA-1, was then cotransfected with a plasmid carrying the hygromycin-resistance gene (pHMR272; Bernard et al., Exp. Cell Res. 158:237-243 (1985)) and either pUHC13-3 or pUHC13-4, resulting in the X and T series of clones, respectively. Clones resistant to hygromycin and G418 were assayed for luciferase activity. As shown in Table 1 below, in the absence of tetracycline, this activity differed in individual

clones by almost four orders of magnitude. However, in all cases, the luciferase activity was sensitive to tetracycline in the culture. This demonstrates that the expression of luciferase is dependent on the function of tTA, which obviously is capable of activating promoter constructs  $P_{hCMV}^*-1$  and  $P_{hCMV}^*-2$ .

5

Table 1. Tetracycline-dependent Luciferase Activity of Different HeLa Cell Clones			
Luciferase activity, rlu/ $\mu$ of protein			
Clone	With Tc	Without Tc	Activation Factor
T7	1074 $\pm$ 75	79,197 $\pm$ 2,119	7.3 $\times 10^1$
T11	2.5 $\pm$ 0.4	34,695 $\pm$ 1,127	1.3 $\times 10^4$
T12	3.5 $\pm$ 0.9	35,298 $\pm$ 5,009	1 $\times 10^4$
T14	$\leq 2$	33 $\pm$ 4	$\geq 1.5 \times 10^1$
T15	286 $\pm$ 47	49,070 $\pm$ 2,784	1.7 $\times 10^2$
T16	$\leq 2$	541 $\pm$ 133	$\geq 2.7 \times 10^2$
X1	$\leq 2$	257,081 $\pm$ 40,137	$\geq 2.7 \times 10^5$
X2	$\leq 2$	104,840 $\pm$ 20,833	$\geq 5 \times 10^4$
X7	75 $\pm$ 7	125,745 $\pm$ 18,204	1.6 $\times 10^3$
The HeLa cell clone HtTA-1, which constitutively expresses tTA, was cotransfected with pUHC13-3 or pUHC13-4 and pHMR272. Hygromycin-resistant clones were examined for luciferase activity. Nine clones identified were subcloned and luciferase activity was quantified in the presence (1 $\mu$ /ml) and absence of tetracycline (Tc). Values are arithmetic means of three independent luciferase determinations (from three independently grown cultures). Luciferase activities of $<2$ rlu/ $\mu$ g of protein are too close to the instrumental background to be quantified.			

When the luciferase activity within various clones was monitored in the presence and absence of tetracycline hydrochloride (Sigma), two remarkable results emerged. (i) In all clones tested, tTA greatly stimulated promoter activity, even up to five orders of magnitude in clone X1. (ii) In clones T14, T16, X1 and X2 (Table 1), tetracycline reduced luciferase activity to values that cannot be quantified even at high protein concentration of extracts due to instrumental limitations (i.e., rlu/ $\mu$ g of protein  $>2$ ). This demonstrates that  $P_{hCMV}^*-1$  and  $P_{hCMV}^*-2$  are virtually silent when integrated in the proper genomic environment and that their activity depends exclusively on the action of tTA.

15

The tTA inactivation studies were carried out with 1  $\mu$ g of tetracycline per ml in the culture medium. A partial inactivation of tTA is, however, readily achieved with tetracycline concentrations below 0.1  $\mu$ g/ml, as shown in Fig. 3, panel a. In the two clones analyzed (T12 and X1), a stepwise reduction of the tetracycline concentration in the medium gradually increased the luciferase activity. These results again demonstrate that, in the case of clone

X1, tTA can regulate transcriptional activity, as monitored by luciferase activity, by over five orders of magnitude. Moreover, at tetracycline concentrations sufficient for full inactivation of tTA (0.1  $\mu\text{g/ml}$ ), no change in growth behavior or morphology of HeLa cells occurs. Only at tetracycline concentrations well above 10  $\mu\text{g/ml}$  were such changes observed upon  
5 prolonged incubation.

#### Kinetics of Tetracycline Action.

The time course of tetracycline action was analyzed in cultures grown in the absence or presence of tetracycline. At time 0, the antibiotic was added to the tetracycline-free  
10 cultures (final concentration, 1  $\mu\text{g/ml}$ ), whereas the tetracycline-containing cultures were rinsed and incubated in fresh antibiotic-free medium (Fig. 3 panel b). At various times, cells were harvested and analyzed for luciferase activity. As shown in Fig. 3 panel b, the depletion of tetracycline leads to a rapid induction of luciferase activity reaching > 20 % of the fully induced level within 12 hr. A similarly rapid reduction of luciferase activity was observed  
15 when tetracycline was added to the fully active tetracycline-free system: within 8 hr activity dropped to about 10 % and reached < 2 % of its original value after 12 hr.

The fusion of the Tn10-derived *E. coli* tetR with the activation domain of VP16 from HSV has generated a transactivator exhibiting all of the properties required for the specific and stringent regulation of an individual gene in a eucaryotic cell. The transactivator tTA  
20 produced in HeLa cells binds specifically to tetO sequences *in vitro*. This association is prevented by tetracycline. When bound to tetOs placed upstream of minimal promoters, tTA efficiently activates transcription from such promoters *in vivo* in a tetracycline-dependent manner. The transactivator is produced in HeLa cells in amounts sufficiently high for strong activation of transcription though low enough to avoid any detectable squelching effects (Gill  
25 & Ptashne Nature (London) 334:721-724 (1988)).

The usefulness of heterologous regulatory systems as the one described here depends decisively on quantitative parameters such as the extent of inactivation and the efficiency of activation of gene expression as well as the kinetics of transition from one state to the other. For the tet system, these parameters were measured in HeLa cell lines that constitutively  
30 express tTA and that also contain the luciferase gene stably integrated and under the control of tTA-dependent promoters. The clones characterized thus far express the luciferase gene to various extents. This is not surprising since differences in the integration sites and in the number of integrated transcription units would be expected. However, in all cases, the expression of luciferase is sensitive to tetracycline. In some clones, tetracycline has the most  
35 dramatic effect of reducing the luciferase activity from high levels over several orders of magnitude to background. This demonstrates that in HeLa cells, the two promoters PhCMV\*-1 and PhCMV\*-2, have no measurable intrinsic activity. Their function strictly depends on tTA. The residual luciferase activity observed in some clones in the presence of tetracycline must therefore be due to position effects.

The tTA-dependent promoters can be kept in a partially activated state by low concentrations of tetracycline. As shown in Fig. 3 panel a, varying the tetracycline concentration between 0 and 0.1  $\mu\text{g/ml}$  allows adjustment of promoter activity within a range of several orders of magnitude. This may allow assessment also of quantitative parameters of gene function *in vivo*.

The activation and inactivation of tTA by the antibiotic appears to be not only an efficient but also a rapid process. When cells from tetracycline containing medium are shifted to tetracycline-free medium, significant luciferase activity is induced within 4 hr and > 20 % of the steady-state level is reached within 12 hr after the shift. Interestingly, even the cultures that were only exposed to tetracycline-free medium during the washing procedure before reincubation in tetracycline-containing medium show a small but reproducible increase in luciferase activity that is still detectable after 4 hr (Fig. 3b).

When tetracycline is added to a culture of X1 cells, luciferase activity is reduced ~ 10-fold within 8 hr and > 50 fold within 12 hr. This decrease is remarkably fast if one takes into account the half-life of luciferase of around 3 hr reported for eucaryotic cells (measured by cycloheximide inhibition: Ilguyen et al., J. Biol. Chem. 264:10487-10492 (1989); Thompson et al., Gene 103:171-177 (1991)) and indicates a rapid uptake of tetracycline by HeLa cells followed by a fast and efficient shutdown of transcription. Although the half-life of luciferase and its mRNA remains to be determined in this system, these conclusions are supported by observations in plant cells, where tetracycline inactivates tetR within < 30 min (Gatz et al., Mol. Gen. Genet 227:229-237 (1991)).

Taken together, these data show that tetracycline, unlike IPTG in a eucaryotic lacR/O-based system, is able to act fast in cultures of eucaryotic cells. The possibility of rapidly switching the activity of a tTA-dependent promoter not only is of interest in studying gene function itself but also should allow analysis of mRNA decay rates of individual genes under physiological conditions.

In clone X1, tetracycline reduces luciferase activity reproducibly by five orders of magnitude. This suggests that binding of tetracycline to tTA may lower the association constant between the transactivator and its operator to a much greater extent than that measured for tetR (Takahasi et al., J. Mol. Biol. 187:341-348 (1986)) and as described for IPTG in the lacR/O system, where the binding constant  $k_{RO}$  is reduced only 1000-fold by the inducer (Barkley and Bourgeois in The Operon, Miller and Reznikoff (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1980; pp. 177-220.)

On the other hand, the results obtained in transient experiments with minimal tk promoters fused to single, dimeric, and heptameric tetO sequences strongly suggest a synergistic effect of multiple tTA binding sites. The efficient inactivation of tTA by tetracycline is therefore most likely due to a large difference in the binding constants of tTA and tTA/tetracycline for the tetO and the nonlinear effect of tetracycline interfering with a cooperative process.

In conclusion, the results indicate that promoter-activating systems as described here are most promising for regulating individual genes in higher eucaryotic cells for several reasons. (i) For activators, in particular when acting through a cooperative mechanism, intracellular concentrations can be kept low, ensuring an efficient inactivation by the effector--in this case, tetracycline. By contrast, repressors in general compete directly with transcription factors and/or RNA polymerases for binding within a promoter region. In the absence of cooperativity, however, the window at which the repressor concentration is sufficiently high for tight expression but still low enough for efficient induction may be narrow and not easily adjustable in different systems. (ii) In an activating system, as described here, the synthesis of tTA can be driven by a tissue-specific promoter, whereas the tTA-dependent promoters are expected to function tissue independently, since they may require only general transcription factors in addition to tTA. By contrast, in a repressor-based system in which operators have to be placed within the context of a promoter sequence, an influence on promoter specificity cannot be excluded. (iii) The tet system offers specific advantages when compared to the intensely studied lac system. For example, tetR binds tetracycline much tighter ( $k_a \sim 10^9 \text{ M}^{-1}$ ; Takahashi et al., J. Mol. Biol. 187:341-348 (1986)) than lacR complexes IPTG ( $k_a 10^6 \text{ M}^{-1}$ ; Barkley & Bourgeois in The Operon, Miller & Reznikoff, eds., Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1980, pp. 177-220). Thus, very low, nontoxic concentrations of tetracycline function effectively. Moreover, a large number of tetracycline analogues are known, of which some appear to have far superior properties as effectors than tetracycline itself. In this context, it is interesting to note that detailed information on the pharmacological properties of tetracycline, in particular pharmacokinetic parameters, is available, which will facilitate application of this system in transgenic animals.

#### **Example 2: Regulation of Gene Expression in Transgenic Animals by tTA**

To examine the ability of tTA to regulate gene expression *in vivo*, transgenic strains of mice were constructed which contained heterologous chromosomal insertions of either a tTA expression unit or a tTA-responsive promoter operably linked to a reporter gene. Single transgenic strains containing either the tTA expression unit or the tTA-responsive reporter unit were then cross bred and double transgenic progeny were identified. The double transgenic animals were then characterized as to the ability of tTA, in a tetracycline dependent manner, to regulate expression of the reporter gene. This example demonstrates that tTA effectively stimulates the expression of a gene operably linked to a tTA responsive promoter in multiple tissues of the animals *in vivo* in the absence of tetracycline (or analogue), whereas expression of the tTA-responsive gene is effectively inhibited in multiple tissues of the animals when tetracycline or an analogue thereof is administered to the animals. These results demonstrate that the tetracycline-controlled transcriptional regulatory system described herein functions effectively in animals, in addition to cell lines *in vitro*.

#### Generation of mice transgenic for a P<sub>h</sub>CMV-tTA expression unit

Mice expressing tTA protein were obtained by pronuclear injection into fertilized oocytes of a 2.7kb XhoI-PfmlI fragment excised from plasmid pUHG15-1. This DNA  
5 fragment contained the tTA gene (shown in SEQ ID NO: 1) under the transcriptional control of the human CMV IE promoter (position +75 to -675) together with a rabbit  $\beta$ -globin polyadenylation site including an intron. The human CMV IE promoter is a constitutive promoter that allows expression of the tetR-VP16 fusion protein in many cell lines where  
10 be functional in a variety of tissues in transgenic mice. DNA was injected into fertilized oocytes at a concentration of approximately 5 ng per  $\mu$ l by standard techniques. Transgenic mice were generated from the injected fertilized oocytes according to standard procedures. Transgenic founder mice were analyzed using polymerase chain reaction (PCR) and Southern hybridization to detect the presence of the tTA transgene in chromosomal DNA of the mice.

15

#### Generation of mice transgenic for the P<sub>h</sub>CMV\*-1 luciferase reporter unit

Mice carrying a P<sub>h</sub>CMV\*-1 luc reporter gene expression unit were generated by pronuclear injection into fertilized oocytes of a 3.1 kb XhoI-EaeI fragment excised from plasmid pUHC13-3. This DNA-fragment contains the luciferase gene under transcriptional  
20 control of the tetracycline-responsive P<sub>h</sub>CMV\*-1 promoter (SEQ ID NO: 5), together with a SV40 t early polyadenylation site including an intron. DNA was injected into oocytes at a concentration of approximately 5 ng per  $\mu$ l and transgenic mice were generated according to standard procedures. Transgenic founder mice were analyzed using Southern hybridization to detect the presence of the P<sub>h</sub>CMV\*-1 luc transgene in chromosomal DNA of the mice.

25

#### Generation of mice transgenic for the P<sub>h</sub>CMV\*-1 luc and P<sub>h</sub>CMV tTA

Having constructed single transgenic mice expressing tTA or carrying P<sub>h</sub>CMV\*-1 luc, double transgenic mice carrying both the tTA expression vector and the luciferase reporter-units were obtained through cross breeding of heterozygous mice transgenic for one  
30 of the two transgenes. Double transgenic animals were identified by standard screenings (e.g., PCR and/or Southern hybridization) to detect the presence of both the tTA transgene and the P<sub>h</sub>CMV\*-1 luc transgene in chromosomal DNA of the mice.

#### Induction and analysis of luciferase activity in tissue samples from mice

35 For oral administration, tetracycline or its derivative doxycycline were given in the drinking water at a concentration of 200  $\mu$ g per ml with 5 % sucrose to hide the bitter taste of the antibiotics. For lactating mice, the concentration was 2 mg per ml with 10 % sucrose to ensure a sufficient uptake via the milk by the young.

To analyze luciferase activity, mice were killed by cervical dislocation and tissue

samples were homogenized in 2 ml tubes containing 500 µl lysis-buffer (25 mM Tris phosphate, pH 7.8/2 mM DTT/ 2 mM EDTA/ 10 % glycerol/ 1 % Triton X100) using a Ultra-Turrax. The homogenate was frozen in liquid nitrogen and centrifuged after thawing for 5 min at 15,000g. 2-20 µl of the supernatant were mixed with 250 µl luciferase assay buffer (25 mM glycylglycine, pH 7.5/ 15 mM MgSO<sub>4</sub>/ 5 mM ATP) and luciferase activity was measured for 10 sec after the injection of 100µl of a 125 µM luciferin solution using Berthold Lumat LB 9501. The protein concentration of the homogenate was determined using Bradford assay and luciferase activity was calculated as relative light units (rlu) per µg of total protein.

## Results

Mice from 4 lines carrying the P<sub>h</sub>CMV-tTA transgene (CT1 through CT4) were mated with mice from line L7, transgenic for P<sub>h</sub>CMV\*<sub>-1</sub> luc. This line shows a very low but significant background of luciferase activity in different organs that is probably due to position effects at the integration side. The luciferase activity in different tissues of the double transgenic mice, either in the presence or absence of the tetracycline analogue doxycycline, is illustrated graphically in Figure 14. High luciferase activity was detectable in five tissues of the double transgenic mice examined: heart, muscle, pancreas, thymus and tongue. The tissue pattern of activated luciferase levels (i.e., in the absence of doxycycline) in the double transgenic mice was similar to expression patterns of the hCMV IE promoter reported in the literature. This is consistent with expression of the luc reporter gene being regulated by tTA (which is expressed in the mice under the control of the hCMV IE promoter). After administration of doxycycline to the mice for 7 days, luciferase activity was reduced close to background levels observed in single transgenic mice carrying only the P<sub>h</sub>CMV\*<sub>-1</sub> luc reporter unit (i.e., the L7 line). Depending on the individual animals used for comparison of induced and non-induced luciferase level, regulation factors up to 10,000 fold can be estimated e.g. in the pancreas. These results indicate that the tetracycline-controlled transcriptional regulatory system described herein can be used to efficiently regulate expression of genes in transgenic animals.

## EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.



- 45 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

5 (A) NAME: BASF Aktiengesellschaft  
(C) CITY: 6700 Ludwigshafen  
(D) STATE: Rheinland-Pfalz  
(E) COUNTRY: Federal Republic of Germany

10 (i) APPLICANT/INVENTORS: Bujard, Hermann  
Gossen, Manfred  
Salfeld, Jochen G.  
Voss, Jeffrey W.

15 (ii) TITLE OF INVENTION: Tight Control of Gene Expression in  
Eucaryotic Cells by Tetracycline-Responsive  
Promoters

(iii) NUMBER OF SEQUENCES: 10

## (iv) CORRESPONDENCE ADDRESS:

20 (A) ADDRESSEE: Lahive & Cockfield  
(B) STREET: 60 State Street  
(C) CITY: Boston  
(D) STATE: Massachusetts  
(E) COUNTRY: USA  
25 (F) ZIP: 02109-1875

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
30 (D) SOFTWARE: ASCII text

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
35 (C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US08/076,327  
(B) FILING DATE: 14-JUN-1993

## (viii) ATTORNEY/AGENT INFORMATION:

40 (A) NAME: DeConti, Giulio A., Jr.  
(B) REGISTRATION NUMBER: 31,503  
(C) REFERENCE/DOCKET NUMBER: BBI-013CPPC

## (ix) TELECOMMUNICATION INFORMATION:

45 (A) TELEPHONE: (617) 227-7400  
(B) TELEFAX: (617) 227-5941

INFORMATION FOR SEQ ID NO:1:

- 46 -

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1008 base pairs  
 (B) TYPE: nucleic acid  
 5 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Herpes Simplex Virus  
 (B) STRAIN: K12, KOS

(vii) IMMEDIATE SOURCE  
 15 (B) CLONE: tTA transactivator

(ix) FEATURE:  
 (A) NAME/KEY: exon  
 (B) LOCATION: 1..1008  
 20

(ix) FEATURE:  
 (A) NAME/KEY: mRNA  
 (B) LOCATION: 1..1008

25 (ix) FEATURE:  
 (A) NAME/KEY: misc. binding  
 (B) LOCATION: 1..207

(ix) FEATURE:  
 30 (A) NAME/KEY: misc. binding  
 (B) LOCATION: 208..335

(ix) FEATURE:  
 35 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1005

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG	48
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40        1                                5                                10                                15	
CTT AAT GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG	96
Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln	
20                                25                                30	
AAG CTA GGT GTA GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG	144
Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys	
35                                40                                45	
CGG GCT TTG CTC GAC GCC TTA GCC ATT GAG ATG TTA GAT AGG CAC CAT	192
Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His	
50                                55                                60	
ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC TGG CAA GAT TTT TTA CGT	240
Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg	
65                                70                                75                                80	

- 47 -

	AAT	AAG	GCT	AAA	AGT	TTT	AGA	TGT	GCT	TTA	CTA	AGT	CAT	CGC	GAT	GGA	288
	Asn	Lys	Ala	Lys	Ser	Phe	Arg	Cys	Ala	Leu	Leu	Ser	His	Arg	Asp	Gly	
					85					90					95		
5	GCA	AAA	GTA	CAT	TTA	GGT	ACA	CGG	CCT	ACA	GAA	AAA	CAG	TAT	GAA	ACT	336
	Ala	Lys	Val	His	Leu	Gly	Thr	Arg	Pro	Thr	Glu	Lys	Gln	Tyr	Glu	Thr	
				100					105					110			
10	CTC	GAA	AAT	CAA	TTA	GCC	TTT	TTA	TGC	CAA	CAA	GGT	TTT	TCA	CTA	GAG	384
	Leu	Glu	Asn	Gln	Leu	Ala	Phe	Leu	Cys	Gln	Gln	Gly	Phe	Ser	Leu	Glu	
			115					120					125				
15	AAT	GCA	TTA	TAT	GCA	CTC	AGC	GCT	GTG	GGG	CAT	TTT	ACT	TTA	GGT	TGC	432
	Asn	Ala	Leu	Tyr	Ala	Leu	Ser	Ala	Val	Gly	His	Phe	Thr	Leu	Gly	Cys	
		130					135					140					
20	GTA	TTG	GAA	GAT	CAA	GAG	CAT	CAA	GTC	GCT	AAA	GAA	GAA	AGG	GAA	ACA	480
	Val	Leu	Glu	Asp	Gln	Glu	His	Gln	Val	Ala	Lys	Glu	Glu	Arg	Glu	Thr	
	145				150					155					160		
25	CCT	ACT	ACT	GAT	AGT	ATG	CCG	CCA	TTA	TTA	CGA	CAA	GCT	ATC	GAA	TTA	528
	Pro	Thr	Thr	Asp	Ser	Met	Pro	Pro	Leu	Leu	Arg	Gln	Ala	Ile	Glu	Leu	
				165					170				175				
30	TTT	GAT	CAC	CAA	GGT	GCA	GAG	CCA	GCC	TTC	TTA	TTC	GGC	CTT	GAA	TTG	576
	Phe	Asp	His	Gln	Gly	Ala	Glu	Pro	Ala	Phe	Leu	Phe	Gly	Leu	Glu	Leu	
			180					185					190				
35	ATC	ATA	TGC	GGA	TTA	GAA	AAA	CAA	CTT	AAA	TGT	GAA	AGT	GGG	TCC	GCG	624
	Ile	Ile	Cys	Gly	Leu	Glu	Lys	Gln	Leu	Lys	Cys	Glu	Ser	Gly	Ser	Ala	
			195					200					205				
40	TAC	AGC	CGC	GCG	CGT	ACG	AAA	AAC	AAT	TAC	GGG	TCT	ACC	ATC	GAG	GGC	672
	Tyr	Ser	Arg	Ala	Arg	Thr	Lys	Asn	Asn	Tyr	Gly	Ser	Thr	Ile	Glu	Gly	
		210					215					220					
45	CTG	CTC	GAT	CTC	CCG	GAC	GAC	GAC	GCC	CCC	GAA	GAG	GCG	GGG	CTG	GCG	720
	Leu	Leu	Asp	Leu	Pro	Asp	Asp	Asp	Ala	Pro	Glu	Glu	Ala	Gly	Leu	Ala	
	225					230					235				240		
50	GCT	CCG	CGC	CTG	TCC	TTT	CTC	CCC	GCG	GGA	CAC	ACG	CGC	AGA	CTG	TCG	768
	Ala	Pro	Arg	Leu	Ser	Phe	Leu	Pro	Ala	Gly	His	Thr	Arg	Arg	Leu	Ser	
				245					250						255		
55	ACG	GCC	CCC	CCG	ACC	GAT	GTC	AGC	CTG	GGG	GAC	GAG	CTC	CAC	TTA	GAC	816
	Thr	Ala	Pro	Pro	Thr	Asp	Val	Ser	Leu	Gly	Asp	Glu	Leu	His	Leu	Asp	
				260					265				270				
60	GGC	GAG	GAC	GTG	GCG	ATG	GCG	CAT	GCC	GAC	GCG	CTA	GAC	GAT	TTC	GAT	864
	Gly	Glu	Asp	Val	Ala	Met	Ala	His	Ala	Asp	Ala	Leu	Asp	Asp	Phe	Asp	
			275					280					285				
65	CTG	GAC	ATG	TTG	GGG	GAC	GGG	GAT	TCC	CCG	GGT	CCG	GGA	TTT	ACC	CCC	912
	Leu	Asp	Met	Leu	Gly	Asp	Gly	Asp	Ser	Pro	Gly	Pro	Gly	Phe	Thr	Pro	
		290					295					300					
70	CAC	GAC	TCC	GCC	CCC	TAC	GGC	GCT	CTG	GAT	ATG	GCC	GAC	TTC	GAG	TTT	960
	His	Asp	Ser	Ala	Pro	Tyr	Gly	Ala	Leu	Asp	Met	Ala	Asp	Phe	Glu	Phe	
	305					310					315					320	

- 48 -

GAG CAG ATG TTT ACC GAT CCC CTT GGA ATT GAC GAG TAC GGT GGG TAG 1008  
 Glu Gln Met Phe Thr Asp Pro Leu Gly Ile Asp Glu Tyr Gly Gly  
                   325                                  330                                  335

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 335 amino acids

(B) TYPE: amino acid

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu  
   1                                  5                                  10                                  15  
 15 Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln  
                                   20                                  25                                  30  
 20 Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys  
                                   35                                  40                                  45  
   Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His  
                                   50                                  55                                  60  
 25 Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg  
                                   65                                  70                                  75                                  80  
   Asn Lys Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly  
                                   85                                  90                                  95  
 30 Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr  
                                   100                                  105                                  110  
   Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu  
                                   115                                  120                                  125  
 35 Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys  
                                   130                                  135                                  140  
 40 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr  
                                   145                                  150                                  155                                  160  
   Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu  
                                   165                                  170                                  175  
 45 Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu  
                                   180                                  185                                  190  
   Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Ala  
                                   195                                  200                                  205  
 50 Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser Thr Ile Glu Gly  
                                   210                                  215                                  220  
 55 Leu Leu Asp Leu Pro Asp Asp Ala Pro Glu Glu Ala Gly Leu Ala

- 49 -

(2) INFORMATION FOR SEQ ID NO:3:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

- 50 -

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5	CTT	AAT	GAG	GTC	GGA	ATC	GAA	GGT	TTA	ACA	ACC	CGT	AAA	CTC	GCC	CAG	96
	Leu	Asn	Glu	Val	Gly	Ile	Glu	Gly	Leu	Thr	Thr	Arg	Lys	Leu	Ala	Gln	
				20				25						30			
	AAG	CTA	GGT	GTA	GAG	CAG	CCT	ACA	TTG	TAT	TGG	CAT	GTA	AAA	AAT	AAG	144
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			35				40						45				
10	CGG	GCT	TTG	CTC	GAC	GCC	TTA	GCC	ATT	GAG	ATG	TTA	GAT	AGG	CAC	CAT	192
	Arg	Ala	Leu	Leu	Asp	Ala	Leu	Ala	Ile	Glu	Met	Leu	Asp	Arg	His	His	
		50				55						60					
15	ACT	CAC	TTT	TGC	CCT	TTA	GAA	GGG	GAA	AGC	TGG	CAA	GAT	TTT	TTA	CGT	240
	Thr	His	Phe	Cys	Pro	Leu	Glu	Gly	Glu	Ser	Trp	Gln	Asp	Phe	Leu	Arg	
	65				70					75						80	
	AAT	AAC	GCT	AAA	AGT	TTT	AGA	TGT	GCT	TTA	CTA	AGT	CAT	CGC	GAT	GGA	288
	Asn	Asn	Ala	Lys	Ser	Phe	Arg	Cys	Ala	Leu	Leu	Ser	His	Arg	Asp	Gly	
				85				90						95			
20	GCA	AAA	GTA	CAT	TTA	GGT	ACA	CGG	CCT	ACA	GAA	AAA	CAG	TAT	GAA	ACT	336
	Ala	Lys	Val	His	Leu	Gly	Thr	Arg	Pro	Thr	Glu	Lys	Gln	Tyr	Glu	Thr	
				100				105						110			
	CTC	GAA	AAT	CAA	TTA	GCC	TTT	TTA	TGC	CAA	CAA	GGT	TTT	TCA	CTA	GAG	384
	Leu	Glu	Asn	Gln	Leu	Ala	Phe	Leu	Cys	Gln	Gln	Gly	Phe	Ser	Leu	Glu	
			115					120					125				
25	AAT	GCA	TTA	TAT	GCA	CTC	AGC	GCT	GTG	GGG	CAT	TTT	ACT	TTA	GGT	TGC	432
	Asn	Ala	Leu	Tyr	Ala	Leu	Ser	Ala	Val	Gly	His	Phe	Thr	Leu	Gly	Cys	
		130				135						140					
30	GTA	TTG	GAA	GAT	CAA	GAG	CAT	CAA	GTC	GCT	AAA	GAA	GAA	AGG	GAA	ACA	480
	Val	Leu	Glu	Asp	Gln	Glu	His	Gln	Val	Ala	Lys	Glu	Glu	Arg	Glu	Thr	
	145				150					155						160	
	CCT	ACT	ACT	GAT	AGT	ATG	CCG	CCA	TTA	TTA	CGA	CAA	GCT	ATC	GAA	TTA	528
	Pro	Thr	Thr	Asp	Ser	Met	Pro	Pro	Leu	Leu	Arg	Gln	Ala	Ile	Glu	Leu	
				165				170						175			
35	TTT	GAT	CAC	CAA	GGT	GCA	GAG	CCA	GCC	TTC	TTA	TTC	GGC	CTT	GAA	TTG	576
	Phe	Asp	His	Gln	Gly	Ala	Glu	Pro	Ala	Phe	Leu	Phe	Gly	Leu	Glu	Leu	
				180				185						190			
	ATC	ATA	TGC	GGA	TTA	GAA	AAA	CAA	CTT	AAA	TGT	GAA	AGT	GGG	TCT	GAT	624
	Ile	Ile	Cys	Gly	Leu	Glu	Lys	Gln	Leu	Lys	Cys	Glu	Ser	Gly	Ser	Asp	
			195				200						205				
40	CCA	TCG	ATA	CAC	ACG	CGC	AGA	CTG	TCG	ACG	GCC	CCC	CCG	ACC	GAT	GTC	672
	Pro	Ser	Ile	His	Thr	Arg	Arg	Leu	Ser	Thr	Ala	Pro	Pro	Thr	Asp	Val	
		210				215						220					
45	AGC	CTG	GGG	GAC	GAG	CTC	CAC	TTA	GAC	GGC	GAG	GAC	GTG	GCG	ATG	GCG	720
	Ser	Leu	Gly	Asp	Glu	Leu	His	Leu	Asp	Gly	Glu	Asp	Val	Ala	Met	Ala	
	225				230					235						240	

- 51 -

```

      CAT GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG      768
      His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly
                245                      250                      255

      GAT TCC CCG GGT CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC      816
5    Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly
                260                      265                      270

      GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG ATG TTT ACC GAT GCC      864
      Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala
                275                      280                      285

10   CTT GGA ATT GAC GAG TAC GGT GGG TTC TAG      894
      Leu Gly Ile Asp Glu Tyr Gly Gly Phe
                290                      295

```

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

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15   (A) LENGTH: 297 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

```

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

20   Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu
      1              5              10              15

      Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln
                20              25              30

25   Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys
      35              40              45

      Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His
30   50              55              60

      Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg
      65              70              75              80

35   Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly
      85              90              95

      Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr
                100              105              110

40   Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu
      115              120              125

      Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys
45   130              135              140

      Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr
      145              150              155              160

50   Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu

```

- 52 -

	165	170	175
	Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu		
	180	185	190
5	Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser Asp		
	195	200	205
	Pro Ser Ile His Thr Arg Arg Leu Ser Thr Ala Pro Pro Thr Asp Val		
10	210	215	220
	Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala		
	225	230	235 240
15	His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Gly Asp Gly		
	245	250	255
	Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly		
	260	265	270
20	Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala		
	275	280	285
	Leu Gly Ile Asp Glu Tyr Gly Gly Phe		
25	290	295	

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 450 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: Human cytomegalovirus  
(B) STRAIN: K12, Towne
- 40 (ix) FEATURE:
- (A) NAME/KEY: mRNA  
(B) LOCATION: 382..450
- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCCTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC	60
ACTCCCTATC AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG	120
AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG	180
TTTACCACTC CCTATCAGTG ATAGAGAAAA GTGAAAGTCG AGTTTACCAC TCCCTATCAG	240
50 TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTATC AGTGATAGAG AAAAGTGAAA	300
GTCGAGCTCG GTACCCGGGT CGAGTAGGCG TGTACGGTGG GAGGCCTATA TAAGCAGAGC	360



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TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG 420  
AAGACACCGG GACCGATCCA GCCTCCGCGG 450

## (2) INFORMATION FOR SEQ ID NO:6:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 450 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:  
(A) ORGANISM: Human cytomegalovirus  
(B) STRAIN: Towne
- 15 (ix) FEATURE:  
(A) NAME/KEY: mRNA  
(B) LOCATION: 382..450

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCCTCG ACCCGGTAC CGAGCTCGAC TTTCACTTTT CTCTATCACT GATAGGGAGT 60  
GGTAAACTCG ACTTTCACCTT TTCTCTATCA CTGATAGGGA GTGGTAAACT CGACTTTCAC 120  
TTTTCTCTAT CACTGATAGG GAGTGGTAAA CTCGACTTTC ACTTTTCTCT ATCACTGATA 180  
GGGAGTGGTA AACTCGACTT TCACTTTTCT CTATCACTGA TAGGGAGTGG TAAACTCGAC 240  
25 TTTCACTTTT CTCTATCACT GATAGGGAGT GGTAAACTCG ACTTTCACCTT TTCTCTATCA 300  
CTGATAGGGA GTGGTAAACT CGAGTAGGCG TGTACGGTGG GAGGCCTATA TAAGCAGAGC 360  
TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG 420  
AAGACACCGG GACCGATCCA GCCTCCGCGG 450

## (2) INFORMATION FOR SEQ ID NO:7:

- 30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 398 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:  
(A) ORGANISM: Herpes Simplex Virus  
(B) STRAIN: KOS
- 40

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCTCGACT TTCACTTTTC TCTATCACTG ATAGGGAGTG GTAAACTCGA CTTTCACCTT 60

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TCTCTATCAC TGATAGGGAG TGGTAAACTC GACTTTTCACT TTTCTCTATC ACTGATAGGG 120  
AGTGGTAAAC TCGACTTTCA CTTTTCTCTA TCACTGATAG GGAGTGGTAA ACTCGACTTT 180  
CACTTTTCTC TATCACTGAT AGGGAGTGGT AAACTCGACT TTCACTTTTC TCTATCACTG 240  
ATAGGGAGTG GTAAACTCGA CTTTCACTTT TCTCTATCAC TGATAGGGAG TGGTAAACTC 300  
5 GAGATCCGGC GAATTCGAAC ACGCAGATGC AGTCGGGGCG GCGCGGTCCG AGGTCCACTT 360  
CGCATATTAA GGTGACGCGT GTGGCCTCGA ACACCGAG 398

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
10 (A) LENGTH: 6244 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: circular
- (ii) MOLECULE TYPE: DNA (genomic)
- 15 (vi) ORIGINAL SOURCE:  
(A) ORGANISM: Human cytomegalovirus  
(B) STRAIN: Towne (hCMV)
- (vii) IMMEDIATE SOURCE:  
(B) CLONE: PUHD BGR3

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT TACCACTCCC 60  
TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACTC CCTATCAGTG ATAGAGAAAA 120  
GTGAAAGTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC 180  
ACTCCCTATC AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG 240  
25 AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG 300  
CTCGGTACCC GGGTCGAGTA GCGGTGTACG GTGGGAGGCC TATATAAGCA GAGCTCGTTT 360  
AGTGAACCGT CAGATCGCCT GGAGACGCCA TCCACGCTGT TTTGACCTCC ATAGAAGACA 420  
CCGGGACCGA TCCAGCCTCC GCGGCCCCGA ATTCGAGCTC GGTACCGGGC CCCCCCTCGA 480  
GGTCGACGGT ATCGATAAGC TTGATATCGA ATTCCAGGAG GTGGAGATCC GCGGGTCCAG 540  
30 CCAAACCCCA CACCCATTTT CTCCTCCCTC TGCCCCATA TCCCGGCACC CCCTCCTCCT 600  
AGCCCTTTCC CTCCTCCCGA GAGACGGGGG AGGAGAAAAG GGGAGTTCAG GTCGACATGA 660  
CTGAGCTGAA GGCAAAGGAA CCTCGGGCTC CCCACGTGGC GGGCGGCGCG CCCTCCCCCA 720  
CCGAGGTCGG ATCCCAGCTC CTGGGTGCGC CGGACCCTGG CCCCTTCCAG GGGAGCCAGA 780  
CCTCAGAGGC CTCGTCTGTA GTCTCCGCCA TCCCCATCTC CCTGGACGGG TTGCTCTTCC 840

	CCCGGCCCTG	TCAGGGGCAG	AACCCCCCAG	ACGGGAAGAC	GCAGGACCCA	CCGTCGTTGT	900
	CAGACGTGGA	GGGCGCATTT	CCTGGAGTCG	AAGCCCCGGA	GGGGGCAGGA	GACAGCAGCT	960
	CGAGACCTCC	AGAAAAGGAC	AGCGGCCTGC	TGGACAGTGT	CCTCGACACG	CTCCTGGCGC	1020
	CCTCGGGTCC	CGGGCAGAGC	CACGCCAGCC	CTGCCACCTG	CGAGGCCATC	AGCCCGTGGT	1080
5	GCCTGTTTGG	CCCCGACCTT	CCCGAAGACC	CCCGGGCTGC	CCCCGCTACC	AAAGGGGTGT	1140
	TGGCCCCGCT	CATGAGCCGA	CCCGAGGACA	AGGCAGGCGA	CAGCTCTGGG	ACGGCAGCGG	1200
	CCCACAAGGT	GCTGCCCAGG	GGACTGTCAC	CATCCAGGCA	GCTGCTGCTC	CCCTCCTCTG	1260
	GGAGCCCTCA	CTGGCCGGCA	GTGAAGCCAT	CCCCGCAGCC	CGCTGCGGTG	CAGGTAGACG	1320
	AGGAGGACAG	CTCCGAATCC	GAGGGCACCG	TGGGGCCGCT	CCTGAAGGGC	CAACCTCGGG	1380
10	CACTGGGAGG	CACGGCGGCC	GGAGGAGGAG	CTGCCCCCGT	CGCGTCTGGA	GCGGCCGCAG	1440
	GAGGCGTCGC	CCTTGTCCTC	AAGGAAGATT	CTCGCTTCTC	GGCGCCAGG	GTCTCCTTGG	1500
	CGGAGCAGGA	CGCGCCGGTG	GCGCCTGGGC	GCTCCCCGCT	GGCCACCTCG	GTGGTGGATT	1560
	TCATCCACGT	GCCCATCCTG	CCTCTCAACC	ACGCTTTCCT	GGCCACCCGC	ACCAGGCAGC	1620
	TGCTGGAGGG	GGAGAGCTAC	GACGGCGGGG	CCGCGGCCGC	CAGCCCCTTC	GTCCCGCAGC	1680
15	GGGGCTCCCC	CTCTGCCTCG	TCCACCCCTG	TGGCGGGCGG	CGACTTCCCC	GA CTGCACCT	1740
	ACCCGCCCCG	CGCCGAGCCC	AAAGATGACG	CGTTCCCCCT	CTACGGCGAC	TTCCAGCCGC	1800
	CCGCCCTCAA	GATAAAGGAG	GAGGAAGAAG	CCGCCGAGGC	CGCGGCGCGC	TCCCCGCGTA	1860
	CGTACCTGGT	GGCTGGTGCA	AACCCCGCCG	CCTTCCCCGA	CTTCCAGCTG	GCAGCGCCGC	1920
	CGCCACCCTC	GCTGCCGCCT	CGAGTGCCCT	CGTCCAGACC	CGGGGAAGCG	GCGGTGGCGG	1980
20	CCTCCCCAGG	CAGTGCCTCC	GTCTCCTCCT	CGTCCCTCGT	GGGGTCGACC	CTGGAGTGCA	2040
	TCCTGTACAA	GGCAGAAGGC	GCGCCGCCCC	AGCAGGGCCC	CTTCGCGCCG	CTGCCCTGCA	2100
	AGCCTCCGGG	CGCCGGCGCC	TGCCTGCTCC	CGCGGGACGG	CCTGCCCTCC	ACCTCCGCCT	2160
	CGGGCGCAGC	CGCCGGGGCC	GCCCCTGCGC	TCTACCCGAC	GCTCGGCCTC	AACGGACTCC	2220
	CGCAACTCGG	CTACCAGGCC	GCCGTGCTCA	AGGAGGGCCT	GCCGCAGGTC	TACACGCCCT	2280
25	ATCTCAACTA	CCTGAGGCCG	GATTCAGAAG	CCAGTCAGAG	CCCACAGTAC	AGCTTCGAGT	2340
	CACTACCTCA	GAAGATTTGT	TTGATCTGTG	GGGATGAAGC	ATCAGGCTGT	CATTATGGTG	2400
	TCCTCACCTG	TGGGAGCTGT	AAGGTCTTCT	TTAAAAGGGC	AATGGAAGGG	CAGCATAACT	2460
	ATTTATGTGC	TGGAAGAAAT	GACTGCATTG	TTGATAAAAT	CCGCAGGAAA	AACTGCCCCG	2520
	CGTGTCGCCT	TAGAAAGTGC	TGTCAAGCTG	GCATGGTCCT	TGGAGGGCGA	AAGTTTAAAA	2580
30	AGTTCAATAA	AGTCAGAGTC	ATGAGAGCAC	TCGATGCTGT	TGCTCTCCCA	CAGCCAGTGG	2640

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	GCATTCCAAA	TGAAAGCCAA	CGAATCACTT	TTTCTCCAAG	TCAAGAGATA	CAGTTAATTC	2700
	CCCCTCTAAT	CAACCTGTTA	ATGAGCATTG	AACCAGATGT	GATCTATGCA	GGACATGACA	2760
	ACACAAAGCC	TGATACCTCC	AGTTCTTTGC	TGACGAGTCT	TAATCAACTA	GGCGAGCGGC	2820
	AACTTCTTTC	AGTGGTAAAA	TGGTCCAAAT	CTCTTCCAGG	TTTTCGAAAC	TTACATATTG	2880
5	ATGACCAGAT	AACTCTCATC	CAGTATTCTT	GGATGAGTTT	AATGGTATTT	GGACTAGGAT	2940
	GGAGATCCTA	CAAACATGTC	AGTGGGCAGA	TGCTGTATTT	TGCACCTGAT	CTAATATTAA	3000
	ATGAACAGCG	GATGAAAGAA	TCATCATTCT	ATTCACATATG	CCTTACCATG	TGGCAGATAC	3060
	CGCAGGAGTT	TGTCAAGCTT	CAAGTTAGCC	AAGAAGAGTT	CCTCTGCATG	AAAGTATTAC	3120
	TACTTCTTAA	TACAATTCCT	TTGGAAGGAC	TAAGAAGTCA	AAGCCAGTTT	GAAGAGATGA	3180
10	GATCAAGCTA	CATTAGAGAG	CTCATCAAGG	CAATTGGTTT	GAGGCAAAAA	GGAGTTGTTT	3240
	CCAGCTCACA	GCGTTTCTAT	CAGCTCACAA	AACTTCTTGA	TAAGTTGCAT	GATCTTGTCA	3300
	AACAACCTCA	CCTGTACTGC	CTGAATACAT	TTATCCAGTC	CCGGGCGCTG	AGTGTGTAAT	3360
	TTCCAGAAAT	GATGTCTGAA	GTTATTGCTG	CACAGTTACC	CAAGATATTG	GCAGGGATGG	3420
	TGAAACCACT	TCTCTTTCAT	AAAAAGTGAA	TGTCAATTAT	TTTTCAAAGA	ATTAAGTGTT	3480
15	GTGGTATGTC	TTTCGTTTTG	GTCAGGATTA	TGACGTCTCG	AGTTTTTATA	ATATTCTGAA	3540
	AGGGAATTCC	TGCAGCCCGG	GGGATCCACT	AGTTCTAGAG	GATCCAGACA	TGATAAGATA	3600
	CATTGATGAG	TTTGACAAA	CCACAAC TAG	AATGCAGTGA	AAAAAATGCT	TTATTTGTGA	3660
	AATTTGTGAT	GCTATTGCTT	TATTTGTAAC	CATTATAAGC	TGCAATAAAC	AAGTTAACAA	3720
	CAACAATTGC	ATTCATTTTA	TGTTTCAGGT	TCAGGGGGAG	GTGTGGGAGG	TTTTTTAAAG	3780
20	CAAGTAAAC	CTCTACAAAT	GTGGTATGGC	TGATTATGAT	CCTGCAAGCC	TCGTCTGCTG	3840
	GCCGGACCAC	GCTATCTGTG	CAAGGTCCCC	GGACGCGCGC	TCCATGAGCA	GAGCGCCCGC	3900
	CGCCGAGGCA	AGACTCGGGC	GGCGCCCTGC	CCGTCCCACC	AGGTCAACAG	GCGGTAACCG	3960
	GCCTCTTCAT	CGGGAATGCG	CGCGACCTTC	AGCATCGCCG	GCATGTCCCC	TGGCGGACGG	4020
	GAAGTATCAG	CTCGACCAAG	CTTGGCGAGA	TTTTTCAGGAG	CTAAGGAAGC	TAAAATGGAG	4080
25	AAAAAATCA	CTGGATATAC	CACCGTTGAT	ATATCCCAAT	GGCATCGTAA	AGAACATTTT	4140
	GAGGCATTTT	AGTCAGTTGC	TCAATGTACC	TATAACCAGA	CCGTTTCACT	GCATTAATGA	4200
	ATCGGCCAAC	GCGCGGGGAG	AGGCGGTTTG	CGTATTGGGC	GCTCTTCCGC	TTCCTCGCTC	4260
	ACTGACTCGC	TGCGCTCGGT	CGTTCCGGCTG	CGGCGAGCGG	TATCAGCTCA	CTCAAAGGCG	4320
	GTAATACGGT	TATCCACAGA	ATCAGGGGAT	AACGCAGGAA	AGAACATGTG	AGCAAAAGGC	4380
30	CAGCAAAAGG	CCAGGAACCG	TAAAAAGGCC	GCGTTGCTGG	CGTTTTTCCA	TAGGCTCCGC	4440

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	CCCCCTGACG	AGCATCACAA	AAATCGACGC	TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA	4500
	CTATAAAGAT	ACCAGGCGTT	TCCCCCTGGA	AGCTCCCTCG	TGCGCTCTCC	TGTTCCGACC	4560
	CTGCCGCTTA	CCGGATACCT	GTCCGCCTTT	CTCCCTTCGG	GAAGCGTGGC	GCTTTCTCAA	4620
	TGCTCACGCT	GTAGGTATCT	CAGTTCGGTG	TAGGTCGTTC	GCTCCAAGCT	GGGCTGTGTG	4680
5	CACGAACCCC	CCGTTTCAGCC	CGACCGCTGC	GCCTTATCCG	GTAACATATCG	TCTTGAGTCC	4740
	AACCCGGTAA	GACACGACTT	ATCGCCACTG	GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA	4800
	GCGAGGTATG	TAGGCGGTGC	TACAGAGTTC	TTGAAGTGGT	GGCCTAACTA	CGGCTACACT	4860
	AGAAGGACAG	TATTTGGTAT	CTGCGCTCTG	CTGAAGCCAG	TTACCTTCGG	AAAAAGAGTT	4920
	GGTAGCTCTT	GATCCGGCAA	ACAAACCACC	GCTGGTAGCG	GTGGTTTTTT	TGTTTGCAAG	4980
10	CAGCAGATTA	CGCGCAGAAA	AAAAGGATCT	CAAGAAGATC	CTTTGATCTT	TTCTACGGGG	5040
	TCTGACGCTC	AGTGGAACGA	AAACTCACGT	TAAGGGATTT	TGGTCATGAG	ATTATCAAAA	5100
	AGGATCTTCA	CCTAGATCCT	TTTAAATTAA	AAATGAAGTT	TTAAATCAAT	CTAAAGTATA	5160
	TATGAGTAAA	CTTGGTCTGA	CAGTTACCAA	TGCTTAATCA	GTGAGGCACC	TATCTCAGCG	5220
	ATCTGTCTAT	TTCGTTTCATC	CATAGTTGCC	TGACTCCCCG	TCGTGTAGAT	AACTACGATA	5280
15	CGGGAGGGCT	TACCATCTGG	CCCCAGTGCT	GCAATGATAC	CGCGAGACCC	ACGCTCACCG	5340
	GCTCCAGATT	TATCAGCAAT	AAACCAGCCA	GCCGGAAGGG	CCGAGCGCAG	AAGTGGTCTC	5400
	GCAACTTTAT	CCGCCTCCAT	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG	AGTAAGTAGT	5460
	TCGCCAGTTA	ATAGTTTGCG	CAACGTTGTT	GCCATTGCTA	CAGGCATCGT	GGTGTACGCG	5520
	TCGTGCTTTG	GTATGGCTTC	ATTCAGCTCC	GGTTCCCAAC	GATCAAGGCG	AGTTACATGA	5580
20	TCCCCCATGT	TGTGCAAAAA	AGCGGTTAGC	TCCTTCGGTC	CTCCGATCGT	TGTCAGAAAGT	5640
	AAGTTGGCCG	CAGTGTATATC	ACTCATGGTT	ATGGCAGCAC	TGCATAATTC	TCTTACTGTC	5700
	ATGCCATCCG	TAAGATGCTT	TTCTGTGACT	GGTGAGTACT	CAACCAAGTC	ATTCTGAGAA	5760
	TAGTGTATGC	GGCGACCGAG	TTGCTCTTGC	CCGGCGTCAA	TACGGGATAA	TACCGCGCCA	5820
	CATAGCAGAA	CTTTAAAAGT	GCTCATCATT	GGAAAACGTT	CTTCGGGGCG	AAAACCTCTCA	5880
25	AGGATCTTAC	CGCTGTTGAG	ATCCAGTTTCG	ATGTAACCCA	CTCGTGCACC	CAACTGATCT	5940
	TCAGCATCTT	TTACTTTTAC	CAGCGTTTCT	GGGTGAGCAA	AAACAGGAAG	GCAAAATGCC	6000
	GCAAAAAAGG	GAATAAGGGC	GACACGGAAA	TGTTGAATAC	TCATACTCTT	CCTTTTTTCAA	6060
	TATTATTGAA	GCATTTATCA	GGGTATTGTG	CTCATGAGCG	GATACATATT	TGAATGTATT	6120
	TAGAAAAATA	AACAAATAGG	GGTTCCGCGC	ACATTTCCCC	GAAAAGTGCC	ACCTGACGTC	6180
30	TAAGAAACCA	TTATTATCAT	GACATTAACC	TATAAAAATA	GGCGTATCAC	GAGGCCCTTT	6240

CGTC

6244

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 4963 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: circular

## (ii) MOLECULE TYPE: DNA (genomic)

## 10 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Human cytomegalovirus

## (vii) IMMEDIATE SOURCE:

(B) CLONE: pUHD BGR4

## 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCGAGTTTA	CCACTCCCTA	TCAGTGATAG	AGAAAAGTGA	AAGTCGAGTT	TACCACTCCC	60
TATCAGTGAT	AGAGAAAAGT	GAAAGTCGAG	TTTACCACTC	CCTATCAGTG	ATAGAGAAAA	120
GTGAAAGTCG	AGTTTACCAC	TCCCTATCAG	TGATAGAGAA	AAGTGAAAGT	CGAGTTTACC	180
ACTCCCTATC	AGTGATAGAG	AAAAGTGAAA	GTGAGTTT	CCACTCCCTA	TCAGTGATAG	240
20 AGAAAAGTGA	AAGTCGAGTT	TACCACTCCC	TATCAGTGAT	AGAGAAAAGT	GAAAGTCGAG	300
CTCGGTACCC	GGGTCGAGTA	GGCGTGTACG	GTGGGAGGCC	TATATAAGCA	GAGCTCGTTT	360
AGTGAACCGT	CAGATCGCCT	GGAGACGCCA	TCCACGCTGT	TTTGACCTCC	ATAGAAGACA	420
CCGGGACCGA	TCCAGCCTCC	GCGGCCCCGA	ATTCCGGCCA	CGACCATGAC	CATGACCCTC	480
CACACCAAAG	CATCTGGGAT	GGCCCTACTG	CATCAGATCC	AAGGGAACGA	GCTGGAGCCC	540
25 CTGAACCGTC	CGCAGCTCAA	GATCCCCCTG	GAGCGGCCCC	TGGGCGAGGT	GTACCTGGAC	600
AGCAGCAAGC	CCGCCGTGTA	CAACTACCCC	GAGGGCGCCG	CCTACGAGTT	CAACGCCGCG	660
GCCGCCGCCA	ACGCGCAGGT	CTACGGTCAG	ACCGGCCTCC	CCTACGGCCC	CGGGTCTGAG	720
GCTGCGGCGT	TCGGCTCCAA	CGGCCTGGGG	GGTTTCCCCC	CACTCAACAG	CGTGTCTCCG	780
AGCCCCTGTA	TGCTACTGCA	CCCGCCGCCG	CAGCTGTCGC	CTTTCCTGCA	GCCCCACGGC	840
30 CAGCAGGTGC	CCTACTACCT	GGAGAACGAG	CCCAGCGGCT	ACACGGTGCG	CGAGGCCGGC	900
CCGCCGGCAT	TCTACAGGCC	AAATTCAGAT	AATCGACGCC	AGGGTGGCAG	AGAAAGATTG	960
GCCAGTACCA	ATGACAAGGG	AAGTATGGCT	ATGGAATCTG	CCAAGGAGAC	TCGCTACTGT	1020
GCAGTGTGCA	ATGACTATGC	TTCAGGCTAC	CATTATGGAG	TCTGGTCCTG	TGAGGGCTGC	1080
AAGGCCCTTCT	TCAAGAGAAG	TATTCAAGGA	CATAACGACT	ATATGTGTCC	AGCCACCAAC	1140
35 CAGTGCACCA	TTGATAAAAA	CAGGAGGAAG	AGCTGCCAGG	CCTGCCGGCT	CCGCAAATGC	1200

	TACGAAGTGG	GAATGATGAA	AGGTGGGATA	CGAAAAGACC	GAAGAGGAGG	GAGAATGTTG	1260
	AAACACAAGC	GCCAGAGAGA	TGATGGGGAG	GGCAGGGGTG	AAGTGGGGTC	TGCTGGAGAC	1320
	ATGAGAGCTG	CCAACCTTTG	GCCAAGCCCG	CTCATGATCA	AACGCTCTAA	GAAGAACAGC	1380
	CTGGCCTTGT	CCCTGACGGC	CGACCAGATG	GTCATGGCCT	TGTTGGATGC	TGAGCCCCCC	1440
5	ATACTCTATT	CCGAGTATGA	TCCTACCAGA	CCCTTCAGTG	AAGCTTCGAT	GATGGGGCTTA	1500
	CTGACCAACC	TGGCAGACAG	GGAGCTGGTT	CACATGATCA	ACTGGGCGAA	GAGGGTGCCA	1560
	GGCTTTGTGG	ATTTGACCCT	CCATGATCAG	GTCCACCTTC	TAGAATGTGC	CTGGCTAGAG	1620
	ATCCTGATGA	TTGGTCTCGT	CTGGCGCTCC	ATGGAGCACC	CAGTGAAGCT	ACTGTTTGCT	1680
	CCTAACTTGC	TCTTGACAG	GAACCAGGGA	AAATGTGTAG	AGGGCATGGT	GGAGATCTTC	1740
10	GACATGCTGC	TGGCTACATC	ATCTCGGTTC	CGCATGATGA	ATCTGCAGGG	AGAGGAGTTT	1800
	GTGTGCCTCA	AATCTATTAT	TTTGCTTAAT	TCTGGAGTGT	ACACATTTCT	GTCCAGCACC	1860
	CTGAAGTCTC	TGGAAGAGAA	GGACCATATC	CACCGAGTCC	TGGACAAGAT	CACAGACACT	1920
	TTGATCCACC	TGATGGCCAA	GGCAGGCCCTG	ACCCTGCAGC	AGCAGCACCA	GCGGCTGGCC	1980
	CAGCTCCTCC	TCATCCTCTC	CCACATCAGG	CACATGAGTA	ACAAAGGCAT	GGAGCATCTG	2040
15	TACAGCATGA	AGTGCAAGAA	CGTGGTGCCC	CTCTATGACC	TGCTGCTGGA	GATGCTGGAC	2100
	GCCCACCGCC	TACATGCGCC	CACTAGCCGT	GGAGGGGCAT	CCGTGGAGGA	GACGGACCAA	2160
	AGCCACTTGG	CCACTGCGGG	CTCTACTTCA	TCGCATTCTT	TGCAAAAGTA	TTACATCACG	2220
	GGGGAGGCAG	AGGGTTTCCC	TGCCACAGTC	TGAGAGCTCC	CTGGCGGAAT	TCGAGCTCGG	2280
	TACCCGGGGA	TCCTCTAGAG	GATCCAGACA	TGATAAGATA	CATTGATGAG	TTTGGACAAA	2340
20	CCACAAC TAG	AATGCAGTGA	AAAAAATGCT	TTATTTGTGA	AATTTGTGAT	GCTATTGCTT	2400
	TATTTGTAAC	CATTATAAGC	TGCAATAAAC	AAGTTAACAA	CAACAATTGC	ATTCAATTTA	2460
	TGTTTCAGGT	TCAGGGGGAG	GTGTGGGAGG	TTTTTTTAAAG	CAAGTAAAAC	CTCTACAAAT	2520
	GTGGTATGGC	TGATTATGAT	CCTGCAAGCC	TCGTCGTCTG	GCCGGACCAC	GCTATCTGTG	2580
	CAAGGTCCCC	GGACGCGCGC	TCCATGAGCA	GAGCGCCCCG	CGCCGAGGCA	AGACTCGGGC	2640
25	GGCGCCCTGC	CCGTCCCACC	AGGTCAACAG	GCGGTAACCG	GCCTCTTCAT	CGGGAATGCG	2700
	CGCGACCTTC	AGCATCGCCG	GCATGTCCCC	TGGCGGACGG	GAAGTATCAG	CTCGACCAAG	2760
	CTTGGCGAGA	TTTTTCAGGAG	CTAAGGAAGC	TAAAATGGAG	AAAAAAATCA	CTGGATATAC	2820
	CACCGTTGAT	ATATCCCAAT	GGCATCGTAA	AGAACATTTT	GAGGCATTTT	AGTCAGTTGC	2880
	TCAATGTACC	TATAACCAGA	CCGTTTCAGCT	GCATTAATGA	ATCGGCCAAC	GCGCGGGGAG	2940
30	AGGCGGTTTG	CGTATTGGGC	GCTCTTCCGC	TTCCTCGCTC	ACTGACTCGC	TGCGCTCGGT	3000

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	CGTTCGGCTG	CGGCGAGCGG	TATCAGCTCA	CTCAAAGGCG	GTAATACGGT	TATCCACAGA	3060
	ATCAGGGGAT	AACGCAGGAA	AGAACATGTG	AGCAAAAGGC	CAGCAAAAGG	CCAGGAACCG	3120
	TAAAAAGGCC	GCGTTGCTGG	CGTTTTTCCA	TAGGCTCCGC	CCCCCTGACG	AGCATCACAA	3180
	AAATCGACGC	TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA	CTATAAAGAT	ACCAGGCGTT	3240
5	TCCCCCTGGA	AGCTCCCTCG	TGCGCTCTCC	TGTTCCGACC	CTGCCGCTTA	CCGGATACCT	3300
	GTCCGCCTTT	CTCCCTTCGG	GAAGCGTGGC	GCTTTCTCAA	TGCTCACGCT	GTAGGTATCT	3360
	CAGTTCGGTG	TAGGTCGTTT	GCTCCAAGCT	GGGCTGTGTG	CACGAACCCC	CCGTTTCAGCC	3420
	CGACCGCTGC	GCCTTATCCG	GTAACATATCG	TCTTGAGTCC	AACCCGGTAA	GACACGACTT	3480
	ATCGCCACTG	GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA	GCGAGGTATG	TAGGCGGTGC	3540
10	TACAGAGTTC	TTGAAGTGGT	GGCCTAACTA	CGGCTACACT	AGAAGGACAG	TATTTGGTAT	3600
	CTGCGCTCTG	CTGAAGCCAG	TTACCTTCGG	AAAAAGAGTT	GGTAGCTCTT	GATCCGGCAA	3660
	ACAAACCACC	GCTGGTAGCG	GTGGTTTTTT	TGTTTGCAAG	CAGCAGATTA	CGCGCAGAAA	3720
	AAAAGGATCT	CAAGAAGATC	CTTTGATCTT	TTCTACGGGG	TCTGACGCTC	AGTGAACGA	3780
	AAACTCACGT	TAAGGGATTT	TGGTCATGAG	ATTATCAAAA	AGGATCTTCA	CCTAGATCCT	3840
15	TTTAAATTAA	AAATGAAGTT	TTAAATCAAT	CTAAAGTATA	TATGAGTAAA	CTTGGTCTGA	3900
	CAGTTACCAA	TGCTTAATCA	GTGAGGCACC	TATCTCAGCG	ATCTGTCTAT	TTCGTTTCATC	3960
	CATAGTTGCC	TGATCCCCGT	CGTGTAGATA	ACTACGATAC	GGGAGGGCTT	ACCATCTGGC	4020
	CCCAGTGCTG	CAATGATACC	GCGAGACCCA	CGCTCACC GG	CTCCAGATTT	ATCAGCAATA	4080
	AACCAGCCAG	CCGGAAGGGC	CGAGCGCAGA	AGTGGTCCTG	CAACTTTATC	CGCCTCCATC	4140
20	CAGTCTATTA	ATTGTTGCCG	GGAAGCTAGA	GTAAGTAGTT	CGCCAGTTAA	TAGTTTGCGC	4200
	AACGTTGTTG	CCATTGCTAC	AGGCATCGTG	GTGTCACGCT	CGTCGTTTGG	TATGGCTTCA	4260
	TTCAGCTCCG	GTTCCCAACG	ATCAAGGCGA	GTTACATGAT	CCCCCATGTT	GTGCAAAAAA	4320
	GCGGTTAGCT	CCTTCGGTCC	TCCGATCGTT	GTCAGAAGTA	AGTTGGCCGC	AGTGTTATCA	4380
	CTCATGGTTA	TGGCAGCACT	GCATAATTCT	CTTACTGTCA	TGCCATCCGT	AAGATGCTTT	4440
25	TCTGTGACTG	GTGAGTACTC	AACCAAGTCA	TTCTGAGAAT	AGTGTATGCG	GCGACCGAGT	4500
	TGCTCTTGCC	CGGCGTCAAT	ACGGGATAAT	ACCGCGCCAC	ATAGCAGAAC	TTTAAAAGTG	4560
	CTCATCATTG	GAAAACGTTT	TTCGGGGCGA	AAACTCTCAA	GGATCTTACCGCT	GTTGAGA	4620
	TCCAGTTCGA	TGTAACCCAC	TCGTGCACCC	AACGTATCTT	CAGCATCTTT	TACTTTCACC	4680
	AGCGTTTCTG	GGTGAGCAAA	AACAGGAAGG	CAAAATGCCG	CAAAAAAGGG	AATAAGGGCG	4740
30	ACACGGAAAT	GTTGAATACT	CATACTCTTC	CTTTTTCAAT	ATTATTGAAG	CATTTATCAG	4800



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GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG 4860  
GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG 4920  
ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTC 4963

## 5 (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGAGTTTAC CACTCCCTAT CAGTGATAGA GAAAAGTGAA AG 42

CLAIMS

1. An isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) at a predetermined location in a second target DNA molecule, the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells, the DNA molecule comprising a polynucleotide sequence encoding the tTA flanked at 5' and 3' ends by additional polynucleotide sequences of sufficient length for homologous recombination between the DNA molecule and the second target DNA molecule at a predetermined location.
2. The DNA molecule of claim 1, wherein the additional polynucleotide sequences flanking the polynucleotide sequence encoding the tTA are of a gene of interest, or regulatory region thereof, into which the DNA molecule is inserted.
3. The DNA molecule of claim 2, wherein, upon integration of the DNA molecule into the gene of interest, or regulatory region thereof, expression of the tTA is controlled by regulatory elements of the gene of interest.
4. The DNA molecule of claim 1, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.
5. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.
6. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.
7. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.
8. The DNA molecule of claim 1, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.

9. The DNA molecule of claim 1, further comprising a polynucleotide sequence encoding a selectable marker.

10. The DNA molecule of claim 9, wherein the nucleotide sequence encoding the selectable marker is a tk gene or a neomycin resistance gene.

11. An isolated DNA molecule for integrating a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter within a predetermined gene of interest in a second target DNA molecule, the DNA molecule comprising:

- a) a first polynucleotide sequence comprising a 5' flanking regulatory region of the gene of interest, operably linked to:
- b) a second polynucleotide sequence encoding a tTA, the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells; and
- c) a third polynucleotide sequence comprising a tTA-responsive promoter, operably linked to:
- d) a fourth polynucleotide sequence comprising at least a portion of a coding region of the gene of interest;

wherein the first and fourth polynucleotide sequences are of sufficient length for homologous recombination between the DNA molecule and the gene of interest in the second target DNA molecule such that expression of the tTA is controlled by 5' regulatory elements of the gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter.

12. The DNA molecule of claim 11, further comprising a fifth polynucleotide sequence encoding a selectable marker operably linked to a regulatory sequence, wherein the fifth polynucleotide sequence is located between the second and third polynucleotide sequences.

13. The DNA molecule of claim 12, wherein the fifth polynucleotide sequence encoding the selectable marker is a tk gene or a neomycin resistance gene.

14. The DNA molecule of claim 11, further comprising a fifth polynucleotide sequence comprising a transcriptional terminator signal, a transcriptional insulator or a matrix attachment region, wherein the fifth polynucleotide sequence is located between the second and third polynucleotide sequences.

15. The DNA molecule of claim 12, further comprising a sixth polynucleotide sequence comprising a transcriptional terminator signal, a transcriptional insulator or a matrix attachment region, wherein the sixth polynucleotide sequence is located between the fifth and third polynucleotide sequences.
- 5 16. The DNA molecule of claim 11, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.
- 10 17. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.
- 15 18. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.
- 20 19. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.
- 25 20. The DNA molecule of claim 11, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.
- 30 21. The DNA molecule of claim 11, wherein the tTA-responsive promoter of the third nucleotide sequence comprises a minimal promoter operably linked to at least one tet operator sequence.
22. The DNA molecule of claim 21, wherein the minimal promoter is derived from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.
- 35 23. The DNA molecule of claim 11, wherein the gene of interest is a human gene.
24. The DNA molecule of claim 23, wherein the human gene encodes a gene product selected from the group consisting of adenosine deaminase, Factor VIII, Factor IX, dystrophin,  $\beta$ -globin, LDL receptor, CFTR, insulin, erythropoietin, anti-angiogenesis factors,

growth hormone, glucocerebrosidase,  $\beta$ -glucouronidase,  $\alpha$ 1-antitrypsin, phenylalanine hydroxylase, tyrosine hydroxylase, ornithine transcarbamylase, arginosuccinate synthetase, UDP-glucuronosyl transferase, apoA1, MDR1, MRP, TNF, soluble TNF receptor, an interleukins, an interferon, a cytokine, a growth factor and a tumor suppressor gene.

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25. A eucaryotic host cell comprising the DNA molecule of claim 1, wherein the DNA molecule is integrated at a predetermined location in a second target DNA molecule in the host cell.

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26. The host cell of claim 25, further comprising a gene of interest operably linked to a tTA-responsive transcriptional promoter.

27. The host cell of claim 26, wherein the tTA-responsive promoter comprises a minimal promoter operably linked to at least one tet operator sequence.

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28. The host cell of claim 27, wherein the minimal promoter is derived from a cytomegalovirus immediate early gene promoter or a herpes simplex virus thymidine kinase gene promoter.

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29. The host cell of claim 25, which is a mammalian cell.

30. The host cell of claim 29, which is a human cell.

31. The host cell of claim 29, which is an embryonic stem cell.

25

32. The host cell of claim 25, which is a yeast cell or a fungal cell.

33. The host cell of claim 25, wherein the cell is an insect cell and the second target DNA molecule is an insect gene or a baculovirus gene.

30

34. A method for inhibiting transcription of the gene of interest operatively linked to the tTA-responsive promoter in the host cell of claim 26, comprising contacting the cell with tetracycline or a tetracycline analogue.

35

35. A host cell comprising the nucleic acid of claim 11, wherein the nucleic acid is integrated into the predetermined gene of interest in a second target DNA molecule in the host cell.

36. The host cell of claim 35, which is a mammalian cell.

37. The host cell of claim 36, which is a human cell.

38. The host cell of claim 36, which is an embryonic stem cell.

39. The host cell of claim 35, which is a yeast cell or a fungal cell.

40. The host cell of claim 35, wherein the cell is an insect cell and the gene of interest is an insect gene or a baculovirus gene.

41. A method for inhibiting transcription of the eucaryotic gene of interest in the host cell of claim 35, comprising contacting the cell with tetracycline or a tetracycline analogue.

42. A non-human transgenic animal having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA), the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells.

43. The animal of claim 42, wherein the Tet repressor of the tTA is a Tn10-derived Tet repressor.

44. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is from herpes simplex virus virion protein 16.

45. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is selected from the group consisting of acidic, proline-rich, serine/threonine-rich and glutamine-rich transcriptional activation polypeptides.

46. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain selected from the group consisting of a leucine zipper domain, a helix-loop-helix domain and a zinc finger domain.

47. The animal of claim 42, wherein the polypeptide of the tTA which directly or indirectly activates transcription in eucaryotic cells is an interaction domain from a TATA binding protein.

48. The animal of claim 42, further having a second transgene comprising a gene of interest operably linked to a tTA-responsive promoter.
49. The animal of claim 42, which is a mouse.
50. The animal of claim 42, which is selected from a group consisting of a cow, a goat, a sheep and a pig.
51. A method for inhibiting transcription of the second transgene in the transgenic animal of claim 48, comprising administering tetracycline or a tetracycline analogue to the animal.
52. A non-human transgenic animal having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA), the tTA comprising a prokaryotic Tet repressor operably linked to a polypeptide which directly or indirectly activates transcription in eucaryotic cells, wherein the transgene is integrated by homologous recombination at a predetermined location within a chromosome within cells of the animal.
53. The animal of claim 52, further having a second transgene comprising a gene of interest operably linked to a tTA-responsive promoter.
54. A method for inhibiting transcription of the second transgene in the animal of claim 53, comprising administering tetracycline or a tetracycline analogue to the animal.
55. A transgenic animal having a transgene comprising a polynucleotide sequence encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter, wherein the transgene is integrated by homologous recombination at a predetermined location within a gene of interest within cells of the animal such that expression of the tTA is controlled by 5' regulatory elements of the gene of interest and expression of the gene of interest is controlled by the tTA-responsive promoter.
56. A method for inhibiting transcription of the gene of interest in the animal of claim 55, comprising administering tetracycline or a tetracycline analogue to the animal.
57. A process for producing and isolating a gene product encoded by the gene of interest operably linked to the tTA-responsive transcriptional promoter in the cell of claim 26, comprising:
- a) growing cells in a culture medium in the presence of tetracycline or a tetracycline analogue;
  - b) reducing the concentration of tetracycline or the tetracycline analogue to stimulate

transcription of the gene of interest;

- c) further culturing the cells until a desired amount of the gene product encoded by the gene of interest is produced by the cells; and
- b) isolating the gene product from harvested cells or from the culture medium.

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58. The process of claim 57, wherein the cells are mammalian cells.

59. The process of claim 57, wherein the cells are yeast or fungal cells.

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60. A method for producing the non-human transgenic animal of claim 42, comprising:  
a) introducing a DNA molecule encoding the tTA into a fertilized oocyte;  
b) implanting the fertilized oocyte in a pseudopregnant foster mother; and  
c) allowing the fertilized oocyte to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.

15

61. A method for producing a host cell having a DNA molecule encoding a tetracycline-controllable transactivator (tTA) integrated at a predetermined location in a second target DNA molecule within the cell, comprising:

a) introducing the DNA molecule of claim 1 into a population of cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the second target DNA molecule; and

b) selecting a cell in which the DNA encoding the tTA has integrated at a predetermined location within the second target DNA molecule.

25

62. A method for producing a non-human transgenic animal having a transgene encoding a tetracycline-controllable transactivator (tTA) integrated at a predetermined location within chromosomal DNA of cells of the animal, comprising:

a) introducing the DNA molecule of claim 1 into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and chromosomal DNA within the cell;

b) selecting an embryonic stem cell in which DNA encoding the tTA has integrated at a predetermined location within the chromosomal DNA of the cell;

c) implanting the embryonic stem cell into a blastocyst;

d) implanting the blastocyst into a pseudopregnant foster mother; and

35

e) allowing the blastocyst to develop into the non-human transgenic animal to thereby produce the non-human transgenic animal.

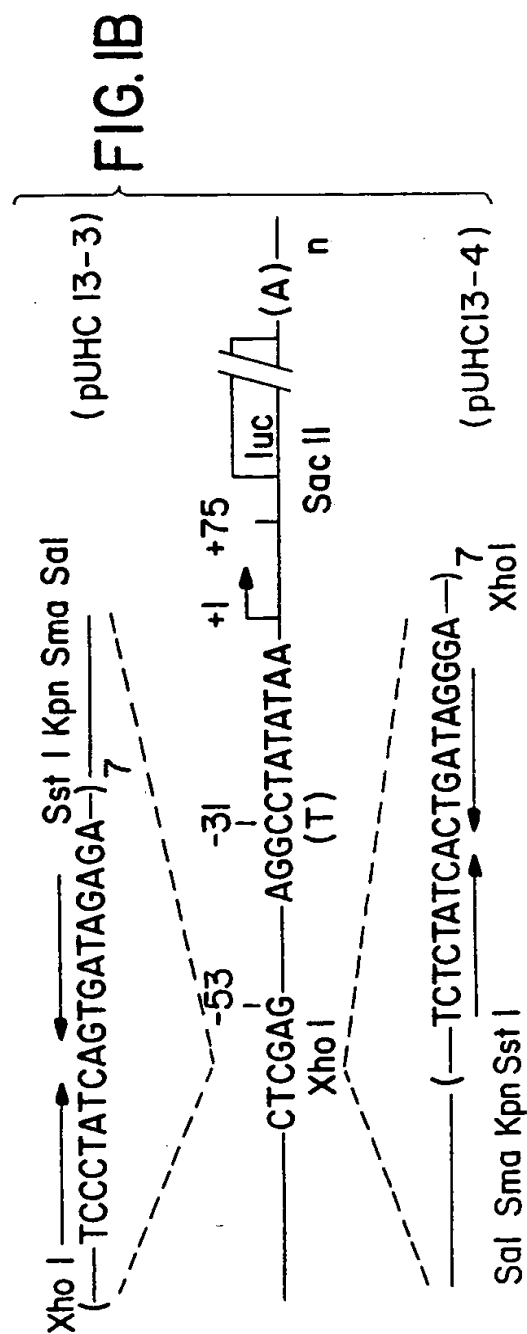
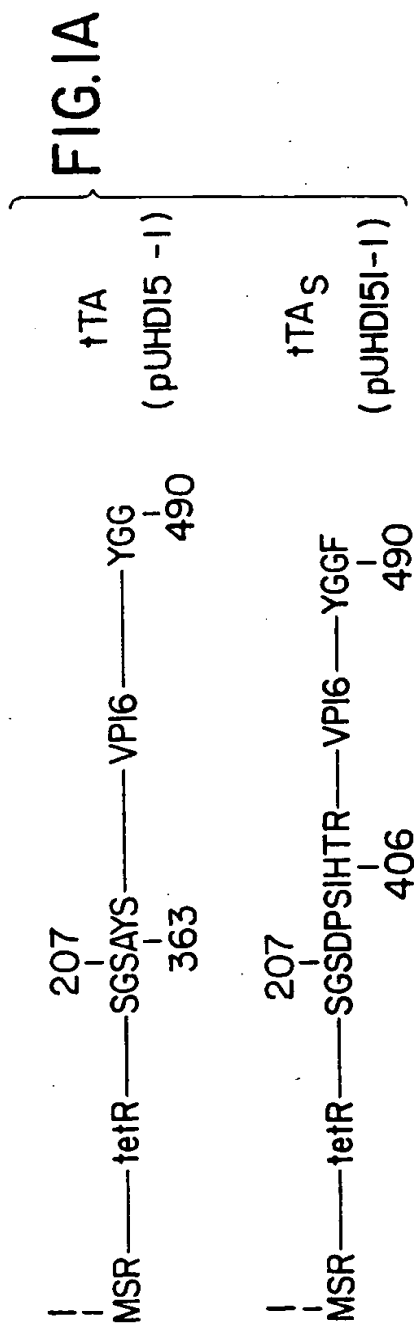
63. A method for producing a non-human transgenic animal having a transgene encoding a tetracycline-controllable transactivator (tTA) and a tTA-responsive promoter integrated at a



predetermined location within a gene of interest in cells of the animal, comprising:

- a) introducing the DNA molecule of claim 11 into a population of embryonic stem cells under conditions suitable for homologous recombination between the DNA encoding the tTA and the gene of interest within the cell;
- 5       b) selecting an embryonic stem cell in which DNA encoding the tTA has integrated at a predetermined location within the gene of interest in the cell;
- c) implanting the embryonic stem cell into a blastocyst;
- d) implanting the blastocyst into a pseudopregnant foster mother; and
- e) allowing the blastocyst to develop into the non-human transgenic animal to thereby
- 10     produce the non-human transgenic animal.

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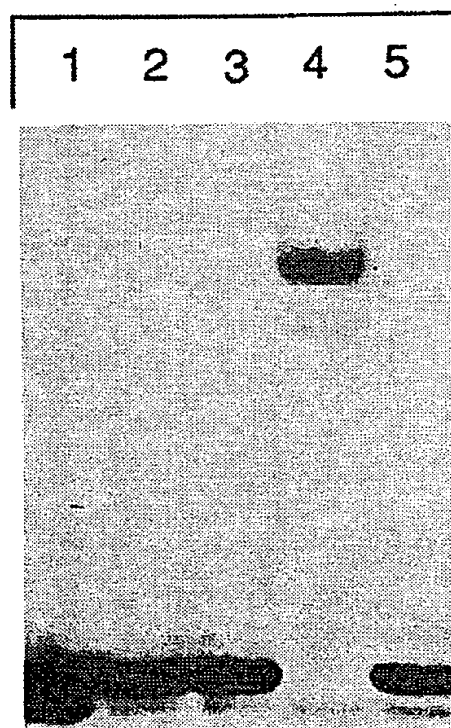


FIG. 2A

FIG. 2B

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FIG. 3B

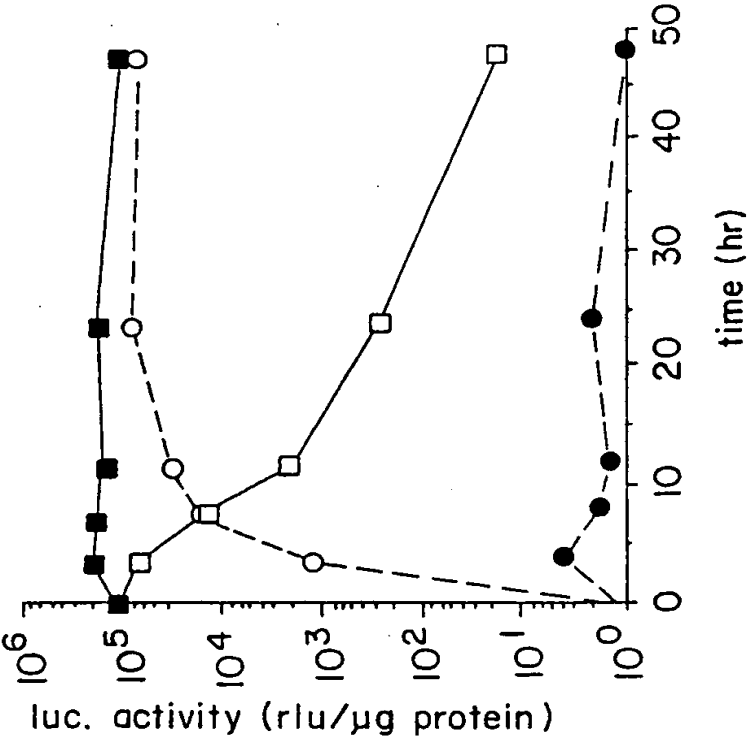
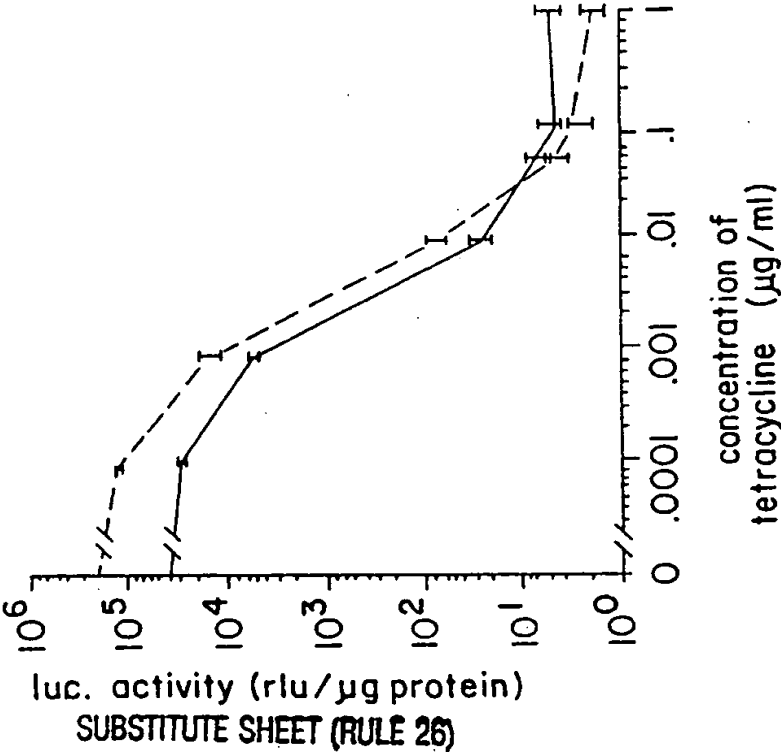


FIG. 3A



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ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT  
Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Asn

GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA  
Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val

GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC  
Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Asp Ala

TTA GCC ATT GAG ATG TTA GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG  
Leu Ala Ile Glu Met Leu Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly

GAA AGC TGG CAA GAT TTT TTA CGT AAT AAG GCT AAA AGT TTT AGA TGT GCT TTA  
Glu Ser Trp Gln Asp Phe Leu Arg Asn Lys Ala Lys Ser Phe Arg Cys Ala Leu

*Fig. 4A*

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CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA  
 Leu Ser His Arg Asp Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys

CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA CAA GGT TTT TCA  
 Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser

CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC  
 Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys

GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA CCT ACT  
 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr Pro Thr

ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA  
 Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln

*Fig. 4B*

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GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA  
Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu

AAA CAA CTT AAA TGT GAA AGT GGG TCC GCG TAC AGC CGC GCG CGT ACG AAA AAC  
Lys Gln Leu Lys Cys Glu Ser Glu Ser Ala Tyr Ser Arg Ala Arg Thr Lys Asn

AAT TAC GGG TCT ACC ATC GAG GGC CTG CTC GAT CTC CCG GAC GAC GCC CCC  
Asn Tyr Gly Ser Thr Ile Glu Gly Leu Leu Asp Leu Pro Asp Asp Ala Pro

GAA GAG GCG GGG CTG GCG GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG  
Glu Glu Ala Gly Leu Ala Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr

CGC AGA CTG TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC  
Arg Arg Leu Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His

*Fig. 4C*

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TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT  
Leu Asp Gly Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp

CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC CAC GAC  
Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp

TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG ATG TTT  
Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Met Phe

ACC GAT CCC CTT GGA ATT GAC GAG TAC GGT GGG TAG  
Thr Asp Pro Leu Gly Ile Asp Glu Tyr Gly Gly \*

*Fig. 4D*

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ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT  
Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Asn

GAG GTC GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA  
Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val

GAG CAG CCT ACA TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC  
Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Asp Ala

TTA GCC ATT GAG ATG TTA GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG  
Leu Ala Ile Glu Met Leu Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly

GAA AGC TGG CAA GAT TTT TTA CGT AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA  
Glu Ser Trp Gln Asp Phe Leu Arg Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu

*Fig. 5A*

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CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA GAA AAA  
 Leu Ser His Arg Asp Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys

CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA CAA GGT TTT TCA  
 Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser

CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT ACT TTA GGT TGC  
 Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys

GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA CCT ACT  
 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr Pro Thr

ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA  
 Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln

*Fig. 5B*

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GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA  
 Gly Ala Glu Pro Ala Phe Leu Phe Gly Ser Gly Leu Glu Ile Ile Cys Gly Leu Glu

AAA CAA CTT AAA TGT GAA AGT GGT TCT GAT CCA TCG ATA CAC ACG CGC AGA CTG  
 Lys Gln Leu Lys Cys Glu Ser Gly Ser Asp Pro Ser Ile His Thr Arg Arg Leu

TCG ACG GCC CCC CCG ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC  
 Ser Thr Ala Pro Pro Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly

GAG GAC GTG GCG ATG GCG CAT GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG  
 Glu Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met

TTG GGG GAC GGG GAT TCC CCG GGT CCG GGA TTT ACC CCC CAC GAC TCC GCC CCC  
 Leu Gly Asp Gly Asp Ser Pro Gly Pro Gly Phe Thr Pro His Asp Ser Ala Pro

*Fig. 5C*

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TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG ATG TTT ACC GAT GCC  
Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe Glu Gln Met Phe Thr Asp Ala

CTT GGA ATT GAC GAG TAC GGT GGG TTC TAG  
Leu Gly Ile Asp Glu Tyr Gly Gly Phe \*

*Fig 5D*

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GAATTCCTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTC  
CCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGT  
GAAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCC  
TATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGA  
AAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTCGGTACCCGGGT  
CGAGTAGGCGGTACGGTGGGAGGCCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGC  
CTGGAGACGCCATCCACGCTGTTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGC  
GG

*Fig. 6*

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GAATTCCTCGACCCGGGTACCGAGCTCGACTTTCACCTTTTCTCTATCACTGATAGGAGTGGTA  
AACTCGACTTTCACCTTTTCTCTATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTTCTCT  
ATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTTCTCTATCACTGATAGGAGTGGTAAA  
CTCGACTTTCACCTTTTCTCTATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTTCTCTAT  
CACTGATAGGAGTGGTAAACTCGACTTTCACCTTTTCTCTATCACTGATAGGAGTGGTAAACT  
CGAGTAGGCGTGTAACGGTGGGAGGCCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGC  
CTGGAGACGCCATCCACGCTGTGTTTGACCTCCATAGAAAGACACCCGGGACCGATCCAGCCTCCGC  
GG

*Fig. 7*

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GAGCTCGACTTTCACCTTTCTCTATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTCTC  
TATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTCTCTATCACTGATAGGAGTGGTAA  
ACTCGACTTTCACCTTTCTCTATCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTCTCTA  
TCACTGATAGGAGTGGTAAACTCGACTTTCACCTTTCTCTATCACTGATAGGAGTGGTAAAC  
TCGACTTTCACCTTTCTCTATCACTGATAGGAGTGGTAAACTCGAGATCCGGCGAATTCCGAAC  
ACGCAGATGCAGTCGGGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCCGTGTGG  
CCTCGAACACCCGAG

*Fig. 8*

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CTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTTACCACCTCCCTATC  
AGTGATAGAGAAAAGTGAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAGT  
CGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTTTACCACCTCCCTATCAG  
TGATAGAGAAAAGTGAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAGTCCG  
AGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAGTCGAGTCGGTACCCGGGTCGAGTA  
GGCGTGTAACGGTGGGAGGCCCTATATAAGCAGAGCTCGTTTAGTGAAACCGTCAGATCGCCTGGAG  
ACGCCATCCACGCTGTTTGTACCTCCATAGAAAGACACCGGACCGATCCAGCCTCCGGGCCCC  
GAATTCGAGCTCGGTACCGGGCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAAT  
TCCAGGAGTGGAGATCCGCGGGTCCAGCCAAACCCACACCCATTCTCTCCTCCTGCCCCC  
TATATCCCGCACCCCTCCTCCTAGCCCTTTCCCTCCTCCCGAGAGACGGGGAGGAGAAAAG  
GGGAGTTCAGGTCGACATGACTGAGCTGAAGGCAAAGGAACCTCGGGCTCCCCACCGTGGCGGGC  
GGCGGCCCTCCCCACCGAGGTCGGATCCCAGCTCCTGGGTGCGCCCGGACCCCTGGCCCCCTTCC  
AGGGGAGCCAGACCCTCAGAGGCCCTCGTCTGTAGTCTCCGCCCATCCCCCATCTCCCTGGACGGGT

*Fig. 9A*

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GCTCTTCCCCGGCCCTGTAGGGCAGAAACCCCCAGACGGAAGACGACGAGACCCACCGTCG  
TTGTACAGCGTGGAGGGCGCATTTCTGGAGTCGAAGCCCGGAGGGGCAGGAGACAGCAGCT  
CGAGACCTCCAGAAAAGGACAGCGCCTGCTGGACAGTGTCTCGACACGCTCCTGGCGCCCTC  
GGTCCCCGGGCAGAGCCACGCCAGCCCTGCCACCTGCGAGGCCATCAGCCCCGTGGTGCCCTGTTT  
GGCCCCGACCTTCCCCGAAGACCCCCGGGTGCCCCCCGCTACCAAAGGGGTGTTGGCCCCGCTCA  
TGAGCCGACCCGAGGACAAGGCAGCGACAGCTCTGGGACGGCAGCGGCCACAAGGTGCTGCC  
CAGGGGACTGTCACCATCCAGGCAGCTGCTGTCTCCCCCTCTGGGAGCCCTCACTGGCCGGCA  
GTGAAGCCATCCCCCGCAGCCCGCTGCGGTGCAGGTAGACGAGGAGGACAGCTCCGAATCCGAGG  
GCACCGTGGGCCCGCTCTGAAGGGCCAACTCGGGCACTGGAGGCA CGCGGCCGAGGAGG  
AGTGCCCCCGTCCGCTCTGGAGCGCGCAGGAGCGTCGCCCTTGTCCTCCAAAGGAAGATTCT  
CGCTTCTCGGCGCCAGGTCTCTTGCGGAGCAGGACGCGCGGTGGCGCCTGGGCGCTCCC  
CGCTGGCCACCTCGGTGTGATTTCATCCACGTGCCCATCTGCCTCTCAACCACTTTCTCT  
GGCCACCCGCACAGCAGCTGTGAGGGGAGAGCTACGACGGCGGGGCCCGCGGCCCGCAGC

*Fig. 9B*

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CCCTTCGTCCTCCGACGCGGGCTCCCTCTCGCTCGTCCACCCCTGTGGCGGGCGGACTTCC  
CCGACTGCACCTACCCGCCCGACGCCGAGCCCAAGATGACGCGTTCCCCCTCTACGGCGGACTT  
CCAGCCGCCGCCCTCAAGATAAAGGAGGAGGAAGAGCCGCGAGGCCGCGCGCTCCCCG  
CGTACGTACCTGGTGGTGCAAAACCCCGCCCTTCCCGGACTTCCAGCTGGCAGCGCCGC  
CGCCACCCCTCGCTGCCGCCCTCGAGTGCCCTCGTCCAGACCCGGGAAGCGCGGTGGCGGCTC  
CCCAGGCAGTGCCCTCCGTCTCCTCGTCCGCGGTGCGACCCCTGGAGTGCACTCCTGTAC  
AAGCAGAAGCGCGCGCCGCCAGCAGGGCCCCCTTCGCGCGCTGCCCTGCAAGCCTCCGGGCG  
CCGGCGCCTGCTCCCGCGGACGGCCTGCCCTCCACCTCCGCCCTCGGGCGCAGCCGCCCG  
GGCCGCCCTGCGCTCTACCCGACGCTCGGCTCAACGGACTCCCGCAACTCGGCTACCAGGCC  
GCCGTGCTCAAGGAGGGCCTGCCGACGCTACACGCCCTATCTCAACTACCTGAGGCCGATT  
CAGAACCCAGTCAGAGCCCAAGTACAGCTTCGAGTCACTACCTCAGAAAGATTGTTGATCTG  
TGGGGATGAAGCATCAGGCTGTCTATGCTGCTCCTCACCTGTGGAGCTGTAAGTCTTCTTT  
AAAAGGCAATGGAAGGCGAGCATAACTATTATGTGCTGGAAGAAATGACTGCATTGTTGATA

*Fig. 9C*

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AAATCCGCAGGAAAACTGCCCGCGGTGTCGCCCTTAGAAAAGTGTGTCAAGCTGGCATGGTTCCT  
TGGAGGGCGAAAGTTTAAAAAGTTCAATAAAGTCAGAGTCATGAGAGCACTCGATGCTGTGCT  
CTCCCACAGCCAGTGGGCATTCCAAAATGAAAGCCAAACGAATCACTTTTCTCCAAGTCAAGAGA  
TACAGTTAATTCCCCCTCTAATCAACCTGTTAATGAGCAATTGAACCAGATGTGATCTATGCAGG  
ACATGACAAACAAAGCCTGATACCTCCAGTTCTTTGCTGACGAGTCTTAATCAACTAGGCGAG  
CGGCAACTTCTTTCAGTGGTAAATGGTCCAAATCTCTTCCAGTTTTCGAAACTTACATATTG  
ATGACCAGATAACTCTCATCCAGTATTCTTGGATGAGTTAATGGTATTTGGACTAGGATGGAG  
ATCCTACAAACATGTCAGTGGGCAGATGCTGTATTTTGCACCTGATCTAATATTAAATGAACAG  
CGGATGAAAGAAATCATTCATTCTACTATGCCCTTACCATGTGGCAGATACCGCAGGAGTTTG  
TCAAGCTTCAAGTTAGCCAAGAAGAGTTCCTCTGCA TGAAAGTATTACTACTTCTTAATACAAT  
TCCTTTGGAAGGACTAAGAAGTCAAAGCCAGTTTGAAGAGATGAGATCAAGCTACATTAGAGAG  
CTCATCAAGGCAATTGGTTTGAGGCCAAAAGGAGTTGTTCCAGCTCACAGCGTTTCTATCAGC  
TCACAAAACCTTCTTGATAACTTGATGATCTTGTCAAACAACCTCACCTGTACTGCCCTGAATAC

*Fig. 9D*

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ATTTATCCAGTCCCGGCGCTGAGTGTGAATTTCCAGAAATGATGTCTGAAGTTATTGCTGCA  
CAGTTACCC AAGATATTGCCAGGATGGTGAAACCACTTCTCTTTCATAAAAAGTGAATGTCAA  
TTATTTTCAAAGAATTAAGTGTGTGGTATGTCTTTTCGTTTTTGGTCAGGATTATGACGTCCTCG  
AGTTTTTATAATATTCTGAAAGGGAATTCTCTGCAGCCCGGGGATCCACTAGTTCTAGAGGATC  
CAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAAATGCAGTGAAAAAATG  
CTTTATTTGTGAAAATTTGTGATGCTATTGCTTTTATTGTAAACCATTAAGCTGCAATAAACAA  
GTTAACAAACAATTGCAATTCATTTTATGTGTTTCAGGTTTCAGGGGAGGTGTGGAGGTTTTTT  
AAAGCAAGTAAACCTCTACAAATGTGGTATGGCTGATTATGATCCTGCAAGCCTCGTCGTCIG  
GCCGGACCACGCTATCTGTGCAAGTCCCCGGACGCGCGCTCCATGAGCAGAGCGCCCCCGCC  
GAGGCAAGACTCGGGCGGCCCTGCCCGTCCCACCAAGGTCAACAGCGGTAAACCGGCCTCTTC  
ATCGGGAATGCGCGGACCTTCAGCATCGCCGGCATGTCCCCCTGGCGGACGGGAAGTATCAGCT  
CGACCAAGCTTGCGGAGATTTTCAGGAGCTAAGGAAGCTAAAAATGGAGAAAAAATCACTGGAT  
ATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTTCAGTCAGTTGC

*Fig. 9E*

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TCAATGTACCTATAACCAGACCGTTTACAGCTGCATTAAATGAATCGGCCAACGCGCGGGAGAGGC  
GGTTTGGGTATTGGGGCGCTCTTCCGGCTTCCCTCGCTCACTGACTCGCTGCGCTCGGTCTGTTCCGGC  
TGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAATCAGGGGATAA  
CGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAGGCCGCGTTG  
CTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGA  
GGTGGGAAACCCGACAGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCG  
CTCTCCTGTTCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTCTCCCTTCGGGAAGCGTG  
GCGCTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGG  
GCTGTGTGCACGAACCCCGTTTCAGCCCGACCGCTGCGCTTATCCGGTAACTATCGTCTTGA  
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA  
GCGAGGTATGTAGCGGTGCTACAGAGTTCTTGAAGTGGTGGCTAACTACGGCTACACTAGAA  
GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAGAGTTGGTAGCTC  
TTGATCCGGCAAAACCAACCGCTGGTAGCGGTGTTTTTTTGTGTTGCAAGCAGCAGATTACG

*Fig. 9F*

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CGCAGAAAAAGGATCTCAAGAAGATCCTTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA  
ACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCT  
TTTAAATTAAAAATGAAAGTTTAAATCAATCTAAAGTATATAGATAAACTTGGTCTGACAGT  
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTG  
CCTGACTCCCCGTCGTGTAGATAACTACGATACGGAGGGCTTACCATCTGGCCCCAGTGCTGC  
AATGATACCGGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGA  
AGGGCCGAGCGCAGAAGTGTCCTGCAACTTTATCCGCCCTCCATCCAGTCTATTAATGTTGCC  
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGG  
CATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTTCAGCTCCGGTTCCTCAACGATCAAGG  
CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTG  
TCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAATCTCTTAC  
TGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAA  
TAGTGTATCGGCGACCGAGTTGCTCTTGCCCGCGTCAATACGGGATAATACCGCGCCACATA

*Fig. 9G*

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GCAGAACTTTAAAAGTGCTCATTTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTT  
ACCGCTGTTGAGATCCAGTTCGATGTAAACCCACTCGTGACACCCAACTGATCTTCAGCATCTTTT  
ACTTTCACCAGCGTTTCTGCGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAA  
GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATATTGAAGCATTTATCA  
GGTTATTGCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGTT  
CCGCGCACATTTCCCCGAAAAGTGCCACCTGACGCTAAGAAACCATTTATTCATGACATTAA  
CCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

*Fig. 9H*

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CTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATC  
AGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGT  
CGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATCAG  
TGATAGAGAAAAGTGAAAGTCGAGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCG  
AGTTTACCACCTCCCTATCAGTGATAGAGAAAAGTGAAAGTCGAGTCGGTACCCGGGTCGAGTA  
GGCGTGACGGTGGGAGGCCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAG  
ACGCCATCCACGCTGTTTGTACCTCCATAGAAAGACACCCGGGACCGATCCAGCCTCCGCGGCCCC  
GAATTCCGGCCACGACCATGACCATGACCCCTCCACACCAAGCATCTGGGATGGCCCTACTGCA  
TCAGATCCAAGGAAACGAGCTGGAGCCCCCTGAACCGTCCGCGAGCTCAAGATCCCCCTGGAGCGG  
CCCCTGGCGGAGGTGTACCTGGACAGCAGCAAGCCCGCCGTGTACAACTACCCCGAGGGCGCCG  
CCTACGAGTTCAACGCCGCGGCCCGCCCAACGCGCAGGTCTACGGTCAGACCGGCCTCCCCCTA  
CGGCCCCGGTCTGAGGCTCGGCGGTTTCGGCTCCAACGGCCTGGGGGTTTCCCCCCTCAAC  
AGCGTGTCTCCGAGCCCCGTGATGTCTACTGCACCCCGCCGCGAGCTGTGCGCTTTCTCTGCAGC

*Fig. 10A*

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CCCACGGCCAGCAGTGCCCTACTACCTGGAGAACGAGCCCAGCGGCTACACGGTGC GCGAGGC  
CGGCCCGCCGCATTCTACAGGCCAAATTCAGATAATCGACGCCAGGGTGGCAGAGAAAGATTG  
GCCAGTACCAATGACAAGGGAAGTATGGCTATGGAATCTGCCAAGGAGACTCGCTACTGTGTCAG  
TGTGCAATGACTATGCTTCAGGCTACCAATTATGGAGTCTGTGTCCTGTGAGGGCTGCAAGGCCTT  
CTTCAAGAGAAGTATTCAAGGACATAACGACTATATGTGTCCAGCCACCAACCAAGTGCACCAT  
GATAAAACAGAGGAAGAGCTGCCAGGCCCTGCCGGCTCCGCAAAATGCTACGAAGTGGGAATGA  
TGAAAGGTGGGATACGAAAAGACCAGAGAGGAGGAATGTTGAAACACAAGCCAGAGAGAGA  
TGATGGGAGGGCAGGGTGAAGTGGGTCTGTCTGGAGACATGAGAGCTGCCAACCTTTGGCCA  
AGCCCGCTCATGATCAAACGCTCTAAGAAGAACAGCCCTGGCCTTGTCCCTGACGGCCGACCAGA  
TGGTCATGGCCTTGTGGATGCTGAGCCCCCCTACTCTATTCCGAGTATGATCCTACCAGACC  
CTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGCTGGTTCACATG  
ATCAACTGGCGGAAGAGGGTGCCAGGCTTTGTGGATTGTGACCCCTCCATGATCAGGTCCACCTTC  
TAGAATGTCCTGGCTAGAGATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGT

*Fig. 10B*

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GAAGCTACTGTTTGCTCCTAAGTCTTGGACAGGAACCCAGGAAAAATGTGTAGAGGGCATG  
GTGGAGATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGCAGGGAG  
AGGAGTTTGTGTGCCCTCAAATCTATTATTTTGCTTAAATCTGGAGTGTACACATTTCTGTCCAG  
CACCTGAAGTCTCTGGAAGAGAAGGACCATATCCACCGAGTCTTGACAAGATCACAGACACT  
TTGATCCACCTGATGGCCAAGGCAGGCCCTGACCTGCAGCAGCACCCAGCGGCTGGCCCCAGC  
TCCTCCTCATCCTCTCCACATCAGGCACATGAGTAACAAGGCATGGAGCATCTGTACAGCAT  
GAAGTCAAGAACGTGGTGCCCCCTCTATGACCTGCTGTGGAGATGCTGGACGCCACCCGCTA  
CATGCGCCCACTAGCCGTGAGGGGCATCCGTGGAGGAGACGGACCAAGCCACTTGGCCACTG  
CGGGCTCTACTTCATCGCATTCCTTGCAAAAGTATTACATCACGGGGGAGGCAGAGGGTTTCCC  
TGCCACAGTCTGAGAGCTCCCTGGCGGAATTCGAGCTCGGTACCCGGGATCCTCTAGAGGATC  
CAGACATGATAAGATACATTGATGAGTTTGACAACCACTAGAAATGCAGTGAAAAAATG  
CTTTATTGTGAAATTGTGATGCTATTGCTTTATTGTAAACCATTAAGCTGCAATAAACAA  
GTTAACAAACAATTGCATTCTTTTATGTTTCAGGTTTCAGGGGAGGTGTGGAGGTTTTTT

*Fig. 10C*

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AAAGCAAGTAAACCTCTACAAATGTGGTATGGCTGATTATGATCCTGCAAGCCTCGTCTGTG  
GCCGGACCACGCTATCTGTGCAAGTCCCCGGACGCGGCTCCATGAGCAGAGCGCCCGCGCC  
GAGGCAAGACTCGGGCGGCCCTGCCCGTCCACCAGGTCAACAGGCGGTAAACGGCCTCTTTC  
ATCGGGAATCGCGCGACCTTCAGCATCGCCGGCATGTCCCCTGGCGGACGGGAAGTATCAGCT  
CGACCAAGCTTGCGGAGATTTTCAGGAGCTAAGGAAGCTAAATGGAGAAAAAATCACTGGAT  
ATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTTCAGTCAGTTGC  
TCAATGTACCTATAACCAGACCGTTTCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGC  
GGTTTGCGTATTGGGCGCTCTTCCGCTTCCCTCGCTCACTGACTCGCTCGCTCGGTCTCGGC  
TGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAA  
CGCAGGAAGAACATGTGAGCAAAGGCCAGCAAAGGCCAGGAACCGTAAAGGCCCGCTTG  
CTGGCGTTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA  
GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCG  
CTCTCCTGTTCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTG

*Fig. 10D*

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GCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGG  
GCTGTGTGCACGAACCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA  
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA  
GCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAA  
GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTC  
TTGATCCGGCAAAACAACCCGCTGGTAGCGGTGGTTTTTTTGTGTGCAAGCAGCAGATTACG  
CGCAGAAAAAAGGATCTCAAGAAGATCCTTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA  
ACGAAAACTCACGTTAAGGATTTTGGTCAATGAGATTATCAAAAAAGGATCTTCACCTAGATCCT  
TTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATAGTAAACTTGGTCTGACAGT  
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTG  
CCTGATCCCCGTCGTGTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGCTGCA  
ATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACCCAGCCAGCCGGAA  
GGGCCGAGCGCAGAAGTGGTCCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAAATTGTTGCCG

*Fig. 10E*

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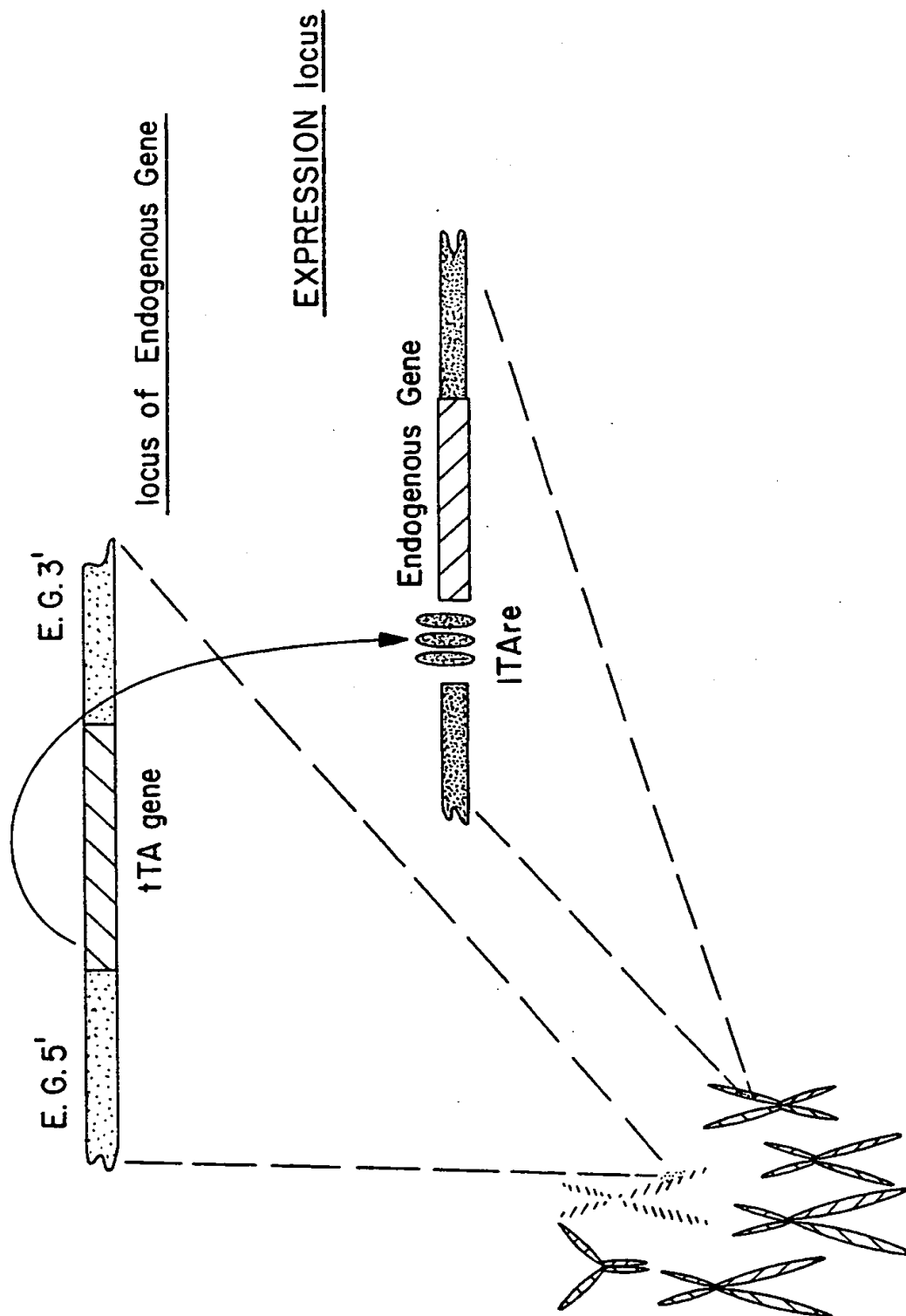
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GGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGGCGCAACGTTGTTGCCATTGCTACAGGC  
ATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTACAGCTCCGGTTCCTCCAAACGATCAAGGC  
GAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGT  
CAGAAAGTAAGTTGGCCGCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAAATTCTCTTACT  
GTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTTCTGAGAAAT  
AGTGATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAG  
CAGAACTTTAAAAGTGCTCATCATTTGAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTA  
CCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCAACCCTGATCTTCAGCATCTTTTA  
CTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAG  
GGCGACACGGAAATGTTGAATACTCATACTCTTCCCTTTTCAATATTATTGAAGCATTTATCAG  
GGTTATTGTCTCATGAGCGGATACATATTGTAAATGTATTTAGAAAAATAAACAAATAGGGGTTTC  
CGCGCACATTTCCCCGAAAGTGCCACCTGACGCTAAGAAACCATTTATTATCATGACATTAAAC  
CTATAAAAATAGGCGTATCACGAGGCCCTTTTCGTC

*Fig. 10F*

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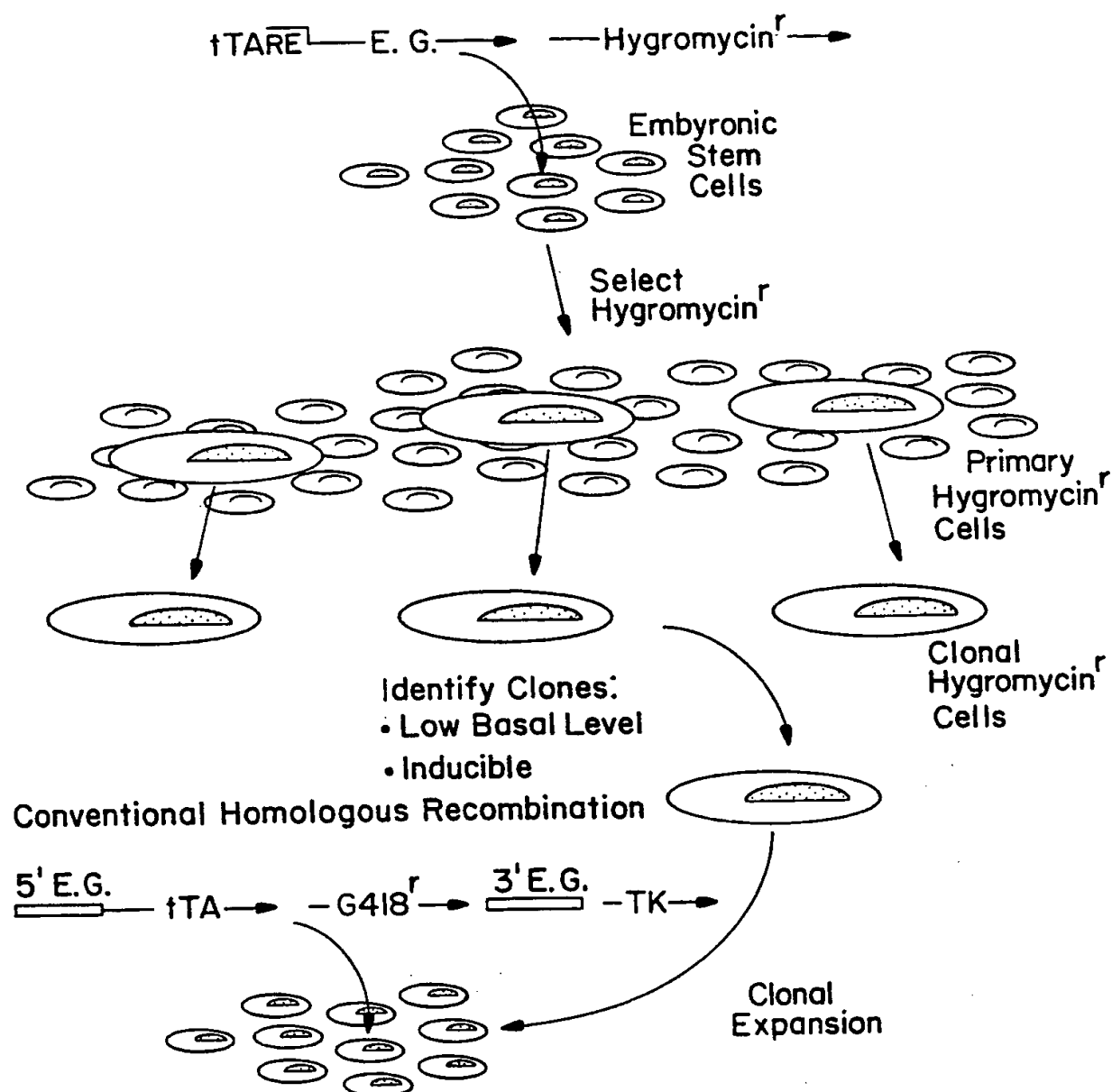
FIG.11



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FIG. 12



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FIG. 13A

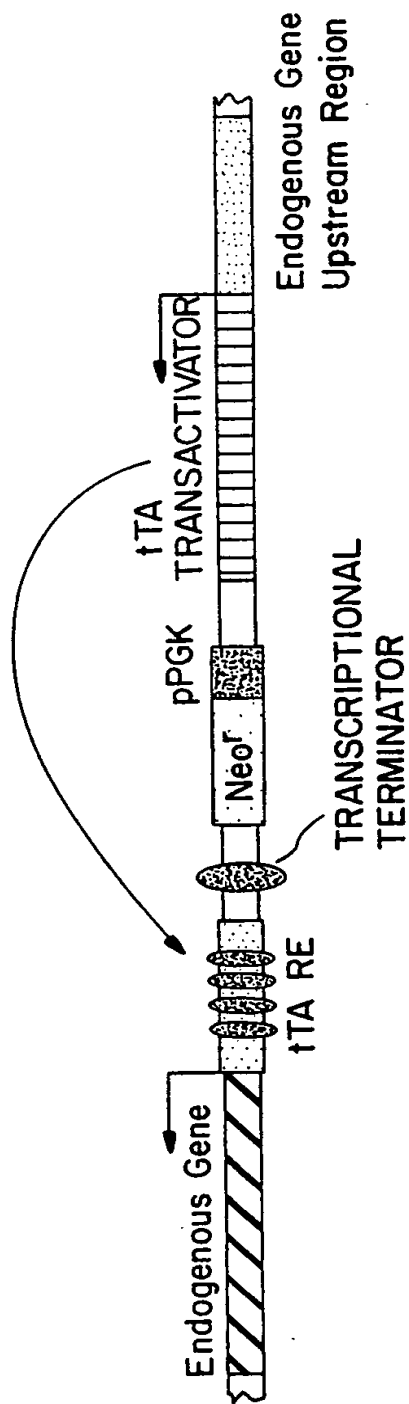
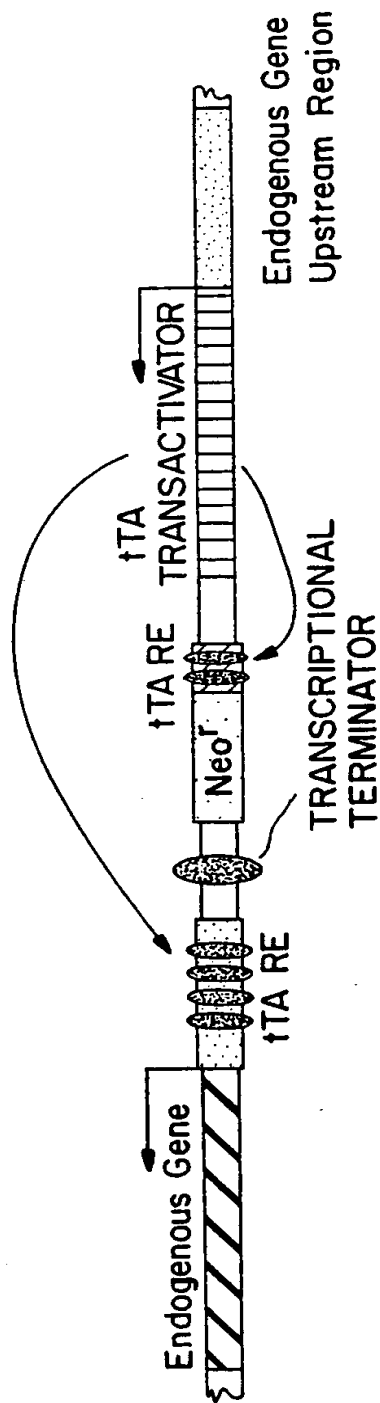


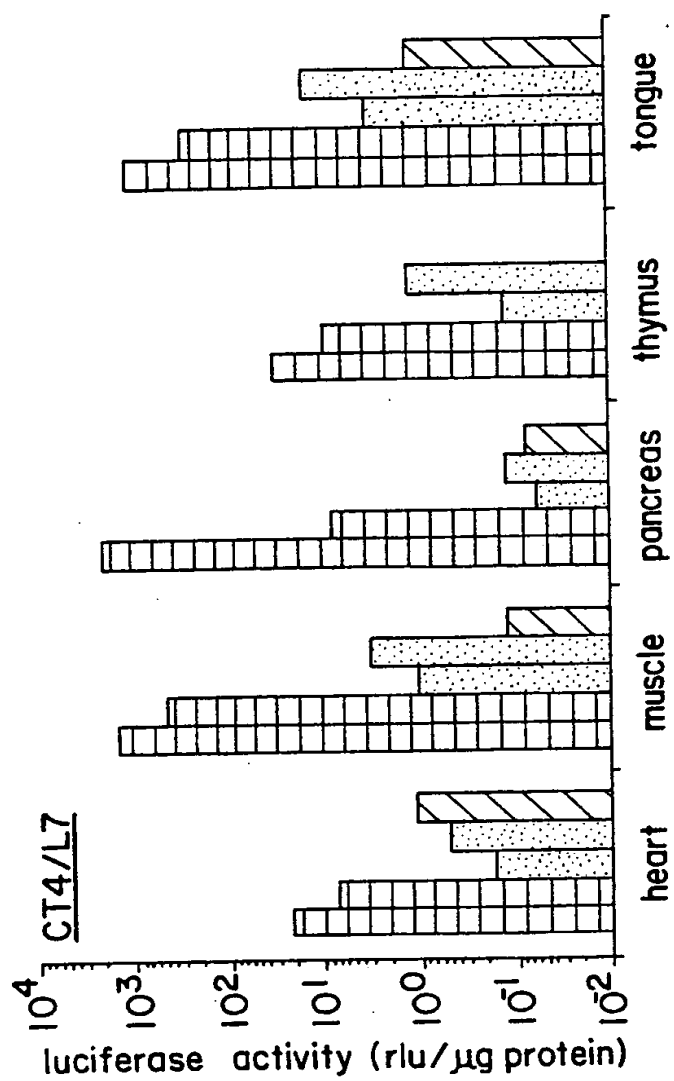
FIG. 13B





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FIG. 14



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